THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

VOLUME 166

CONTENTS OF VOLUME 166

No. 1, November, 1946

No. 2, December, 1946

Letters to the Editors

PREPARATION OF d-TYROSINE, ITS ACETYL DERIVATIVES, AND d-3,4-DIHYDROXYPHENYLALANINE*

By ROBERT RIDGELY SEALOCK

(From the Department of Chemistry, Iowa State College, Ames)

In the continued investigation of the rôle of ascorbic acid in the metabolism of tyrosine (1), relatively large amounts of the unnatural isomer of the amino acid, the acetyl derivatives, and the unnatural 3,4-dihydroxyphenylalanine were required. The preparation of d-tyrosine by resolution of the synthetic benzoyl derivative has been described by Fischer (2). Later Abderhalden and Sickel (3) reported the resolution of the formyl derivative of tyrosine racemized by extensive treatment with strong sodium hydroxide. Since the acetyl compound was also desired, the convenient catalytic racemization procedure described by du Vigneaud and Meyer (4) should afford ready access to the inactive compound. Racemization in this case is accomplished by treatment of the sodium salt of the amino acid in aqueous solution with an excess of acetic anhydride, diacetyl-dl-tyrosine being obtained. This method is of additional value, for it permits the use of the readily available natural isomer.

In preliminary tests the diacetyl-dl-tyrosine failed to yield satisfactory crystalline salts with l-brucine. In contrast, the N-monoacetyl-dl-tyrosine in absolute alcohol gave excellent yields of the salt of the dextro enantiomorph, a high degree of purity being obtained in the first crystallization. The preparation of the monoacetyl compound was accomplished by dissolving the diacetyl derivative in sufficient sodium hydroxide to make the solution more alkaline than pH 8.5, as described by Herriott (5). Consequently, in the method finally evolved, the isolation of the diacetyl derivative was omitted, the acetylation-racemization reaction being followed (after distillation of the excess acetic acid) with alkaline hydrolysis. N-Acetyl-dl-tyrosine was then isolated in 90 to 95 per cent yield.

The crystalline brucine salt after three or four recrystallizations from alcohol exhibited maximum rotation and was subsequently decomposed. The acetyl-d-tyrosine so obtained was then used as such or hydrolyzed to d-tyrosine. Calculated on the basis of the racemic acetyl derivative used, over-all yields of 70 per cent were observed.

From the mother liquors of the resolution procedure the brucine salt of

---

1

the levo isomer may be obtained by evaporation of the alcohol and crystallization of the residue from water. Such a procedure is of value only in special circumstances, as, for example, when excess of isotopic atoms have been introduced into the tyrosine molecule. In practice it has been found expedient merely to recover the tyrosine from the above mother liquors for purpose of reracemization and resolution.

Preparation of the additional acetyl derivatives of *l*- and *d*-tyrosine was accomplished by standard procedures. N-Acetyl-*l*-tyrosine was made by the method described by du Vigneaud and Meyer (4), in which method the solution is maintained alkaline throughout the course of the reaction. The diacetyl-*l*- and diacetyl-*d*-tyrosine were prepared according to the directions of Bergmann and Zervas (6) for the levo compound. However, consideration of the various procedures employed led to interesting conclusions concerning the acetylation of tyrosine. As du Vigneaud and Meyer (4) have demonstrated, the catalytic racemization of α-amino acids occurs in weakly acid solution in the presence of sodium acetate and acetic anhydride. In the case of tyrosine the racemic diacetyl derivative is formed. In contrast, when the acetylation is performed in the presence of excess sodium hydroxide, the optically active N-monoacetyl compound is obtained as likewise described by these authors. The production of the diacetyl in the one case and the monoacetyl in the other is understandable on the basis of the method of Herriott (5), for in the more alkaline solution the O-acetyl group is removed as fast as it is formed.

If this is true, then one would predict that the preparation of either the diacetyl-*l* or diacetyl-*d* compound is best carried out by acetylating tyrosine with rigid control of the pH. By constantly maintaining the pH between 6.0 and 8.0, the diacetyl derivative should be obtained without racemization occurring. This proved to be the case, for several experiments in which the pH was maintained between these limits resulted in the production of the desired isomer completely free of the opposite form.

For the sake of convenience a tabulation of the *l*- and *d*-tyrosines and their respective acetyl derivatives is presented. The bibliographic reference numbers in the final column (Table I) indicate previous descriptions of these compounds.

The availability of *d*-tyrosine also has made it possible to prepare *d*-3,4-dihydroxyphenylalanine by the method used by Waser and Lewandowski (9) for the levo isomer. In this method tyrosine was nitrated in the 3 position, reduced, diazotized, and subsequently converted to the dihydroxyamino acid. By this means *d*-tyrosine likewise gave satisfactory yields of the desired dextro enantiomorph which agreed in its properties with those described by Harington and Randall (10). These authors obtained the unnatural isomer by resolution of the synthetic compound.

Preparation of N-Acetyl-dl-tyrosine—100 gm. of *l*-tyrosine were dissolved in 1120 ml. of N sodium hydroxide and 400 ml. of redistilled acetic anhydride were added in ten portions at 10 minute intervals with continuous stirring. The reaction mixture was allowed to stand in a water bath at 60–70° for 6 hours, after which the calculated amount of 6 N sulfuric acid exactly to neutralize the sodium hydroxide was added. In order to remove the acetic acid, this solution was distilled to a thick syrup *in vacuo* and again concentrated with two portions of water. The material soluble in wet acetone, after removal of the acetone by distillation *in vacuo*, was dissolved

TABLE I

Tyrosine and Acetyltyrosine Derivatives

All melting points are corrected. Optical activity was measured with the D line of sodium. Following the specific rotation are the items of concentration, solvent, and temperature respectively.

Derivative	Formula	Mol. wt.	Melting point	Optical rotation	Nitrogen analysis Calculated	Found	Bibliographic reference No.
			°C.	degrees	per cent	per cent	
l-Tyrosine	$C_9H_{11}O_3N$	181		−10.6 (4%, N HCl, 22°)	7.73	7.74	
d-Tyrosine	"	181		+10.3 (4% " " 25°)	7.73	7.71	
N-Acetyl-*dl*- (monohydrate).	$C_{11}H_{13}O_4N \cdot H_2O$	241	94– 95	0	5.81	5.85	
N-Acetyl-*l*-	$C_{11}H_{13}O_4N$	223	153–154	+47.3 (0.5%, H_2O, 26°)	6.28	6.33	(4, 7)
N-Acetyl-*d*-	"	223	153–154	−48.3 (0.5%, " 25°)	6.28	6.33	
Diacetyl-*dl*-	$C_{13}H_{15}O_5N$	265	168–170	0	5.28	5.22	(4)
Diacetyl-*l*-	"	265	171–172	+40.4 (0.5%, H_2O, 27°)	5.28	5.19	(6, 8)
Diacetyl-*d*-	"	265	171–172	−38.7 (0.5%, " 26°)	5.28	5.13	

in 100 ml. of water for purpose of hydrolysis of the acetoxy group. 400 ml. of 2 N sodium hydroxide were added to adjust the reaction to pH 10 to 11. After 30 minutes at room temperature the calculated amount of 6 N sulfuric acid was added, and the mixture concentrated to a thick syrup. The acetyltyrosine was separated from sodium sulfate with wet acetone, and then crystallized from 300 ml. of water, 116 gm. being obtained. After concentration of the mother liquor and the removal of a second crop of crystals, the combined material weighed 120 gm. Samples dried *in vacuo* over phosphorus pentoxide at 65° lost 7.60 and 7.68 per cent respectively, whereas 1 molecule of water of hydration is calculated as 7.47 per cent. A yield of 90.3 per cent was thus obtained.

Resolution of Acetyl-dl-tyrosine—54.1 gm. of acetyl-*dl*-tyrosine monohydrate and an equimolar quantity (88.5 gm.) of anhydrous *l*-brucine were dissolved in 550 ml. of boiling absolute alcohol. The 75 gm. (54 per cent of the total amount) of impure brucine salt of acetyl-*d*-tyrosine which separated after remaining overnight or longer in the refrigerator were then recrystallized three or four times from 5 volumes of 95 per cent alcohol. From repeated resolutions in this fashion the pure salt was obtained in average yields of 75 per cent of the theoretical, calculated on the basis of anhydrous material. The air-dried material melting at 150–155° (shrinking at 130°) when further dried *in vacuo* over phosphorus pentoxide at 100° was found to decrease from 7.85 to 10.6 per cent in weight. Consequently, all samples for analytical purposes were dried to constant weight under the above conditions. A 0.5 per cent solution of the anhydrous salt in 95 per cent alcohol gave $[\alpha]_D^{26} = -42.3°$. Nitrogen analysis gave 6.76 per cent, as compared to the calculated value of 6.80 per cent.

For purposes of reference, the brucine salt of acetyl-*l*-tyrosine was prepared. The original mother liquor was evaporated to dryness *in vacuo*, the alcohol being completely removed by three distillations with small portions of water. The residue was then recrystallized from 5 volumes of water. After two recrystallizations, the air-dried material, m.p. 110–112° with shrinking at 94°, represented 74 per cent of the theoretical and contained 5.8 per cent moisture. A 1 per cent solution of the anhydrous salt in alcohol gave $[\alpha]_D^{25} = +10.7°$.

Ordinarily the mother liquors obtained from the recrystallization of the *d* salt are used only for the purpose of recovering the brucine and tyrosine. The brucine salt is decomposed and the acetyltyrosine solution treated with hydrochloric acid. The free tyrosine is again subjected to the racemization procedure.

Preparation of Acetyl-d-tyrosine—The brucine salt of acetyl-*d*-tyrosine was dissolved in 20 volumes of warm water and, after quickly cooling to approximately 40°, 2 N sodium hydroxide was added until the reaction was alkaline to phenolphthalein. The brucine was removed by filtration after 12 hours in the cold and thoroughly washed with cold water. The combined alkaline filtrates were extracted five times with chloroform to remove traces of brucine. After addition of the calculated amount of 6 N sulfuric acid and evaporation to dryness, the acetone-soluble material was crystallized from 100 ml. of water. From 88 gm. of brucine salt 22 gm. of acetyl-*d*-tyrosine representing a 69 per cent yield were obtained. The mother liquor may be concentrated to obtain additional material, although in practice it has been preferable to hydrolyze the residue to free *d*-tyrosine.

d-Tyrosine—The unnatural isomer has been obtained by suspending the crystalline acetyl derivative in 10 volumes of 5 N hydrochloric acid and

gently refluxing for 2.5 hours. The excess acid was removed by concentrating *in vacuo*, and redistilling to dryness with three portions of water. The residue was dissolved in water, treated with norit, and free *d*-tyrosine precipitated by the addition of excess sodium hydroxide and sufficient acetic acid to make the solution just acid to litmus. By this method pure *d*-tyrosine is obtained in 93 per cent yield. The over-all yield from the original tyrosine is thus 62 to 66 per cent.

Diacetyl-d-tyrosine—Since acetylation of *l*-tyrosine in alkaline solution (pH > 8.5) yields N-monoacetyl-*l*-tyrosine and in acid solution (excess acetic anhydride) yields diacetyl-*dl*-tyrosine, the following method was used to prepare either the dextro or levo diacetyl derivative.

In a beaker placed in an ice bath, 15 gm. of *d*-tyrosine were dissolved in 42.5 ml. of 2 N sodium hydroxide and 25 ml. of water. In the solution were suspended the extension electrodes of a Beckman pH meter and a motor stirrer. From a burette 24.0 ml. of acetic anhydride were added dropwise. From a second burette 2 N sodium hydroxide was also added dropwise and at such a rate that the reaction of the mixture was maintained between pH 6 and 8. The ice bath was then removed and the reaction mixture allowed to stand at room temperature for 30 minutes. 6 N sulfuric acid exactly to neutralize the sodium hydroxide was added, and the filtered solution immediately taken to dryness *in vacuo*. The diacetyl-*d*-tyrosine was obtained in crystalline form by acetone extraction and solution in water. 16.5 gm. (75 per cent), melting at 172°, were obtained.

In the case of the levo isomer, this method yielded a final product which exhibited the properties described by Bergmann and Zervas (6) and Bergmann and Stern (8).

d-3,4-Dihydroxyphenylalanine—*d*-Tyrosine was nitrated in the 3 position by the method of Waser and Lewandowski (9), the free amino acid being obtained in maximum yields of 55 per cent. At slightly lower temperature of reaction Johnson and Kohmann (11) have reported 65 per cent yields. In the subsequent reduction and conversion to the hydroxy compound (9) maximum yields of 68 and 85 per cent respectively have been obtained. Over-all yields of 32 to 38 per cent are thus readily achieved.

SUMMARY

The preparation of *d*-tyrosine from the readily available natural isomer has been accomplished by catalytic racemization with acetic anhydride in aqueous solution and resolution of the N-acetyl-*dl* compound with brucine.

A method of preparing the diacetyl-*l*-tyrosine or diacetyl-*d*-tyrosine is described. By use of continuous pH control, racemization on the one hand and formation of the monoacetyl derivative on the other have been avoided.

The acetyl derivatives of tyrosine and certain characteristic properties of them have been tabulated.

From *d*-tyrosine, *d*-3,4-dihydroxyphenylalanine has been prepared by methods previously recorded in the literature for the preparation of the natural isomer.

BIBLIOGRAPHY

1. Lan, T. H., and Sealock, R. R., *J. Biol. Chem.*, **155**, 483 (1944).
2. Fischer, E., *Ber. chem. Ges.*, **32**, 3638 (1899).
3. Abderhalden, E., and Sickel, H., *Z. physiol. Chem.*, **131**, 277 (1923).
4. du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98**, 295 (1932).
5. Herriott, R. M., *J. Gen. Physiol.*, **19**, 283 (1935).
6. Bergmann, M., and Zervas, L., *Biochem. Z.*, **203**, 280 (1928).
7. Takenaka, Y., *Acta schol. med. univ. imp. Kioto*, **4**, 367 (1922).
8. Bergmann, M., and Stern, F., *Ber. chem. Ges.*, **63**, 437 (1930).
9. Waser, E., and Lewandowski, M., *Helv. chim. acta*, **4**, 657 (1921).
10. Harington, C. R., and Randall, S. S., *Biochem. J.*, **25**, 1029 (1931).
11. Johnson, T. B., and Kohmann, E. F., *J. Am. Chem. Soc.*, **37**, 1863 (1915).

STRUCTURAL SPECIFICITY OF TYROSINE IN RELATION TO THE METABOLIC ACTION OF ASCORBIC ACID*

By DANIEL H. BASINSKI† AND ROBERT RIDGELY SEALOCK‡

(*From the Department of Vital Economics, The University of Rochester, Rochester, New York*)

In previous publications, it has been established that the metabolic handling of the amino acids phenylalanine and tyrosine by the guinea pig is dependent on an adequate intake of ascorbic acid (1, 2). The premature infant exhibits the same dependence upon a sufficient quantity of the vitamin, since in a state of deficiency intermediate compounds in the oxidation of these aromatic amino acids appear in the urine, as Levine and his associates (3, 4) have demonstrated. Phenylpyruvic acid follows the same metabolic pathway as the amino acids when fed to the vitamin C-deficient guinea pig (2). In contrast, *p*-hydroxyphenylpyruvic acid is readily metabolized in spite of a marked state of scurvy (2).

This striking and unexpected difference in the metabolic behavior of such closely related compounds suggests that the step in the metabolic chain of events at which vitamin C exerts its effect may be determined by the use of properly substituted phenylalanine and tyrosine derivatives and intermediates.

In the experiments described here, the immediate objective has been the modification, one at a time, of each characteristic feature of the original amino acid structure and the subsequent evaluation of metabolic behavior in the scorbutic guinea pig. The specificity of the levo configuration of the *α*-carbon has been studied by feeding the unnatural isomers, *d*-phenylalanine and *d*-tyrosine. The importance of the free amine group has been tested by using the N-acetyl derivatives of the natural or levo forms. For further comparison diacetyl-*l*-tyrosine has been included. Feeding of *p*-methoxyphenylalanine, containing the highly stable methyl ether linkage, has permitted examination of the rôle of the para position in the aromatic nucleus. The influence of modification of the alanine side chain has been determined by feeding the next higher homologue, phenylaminobutyric

acid, and the thiomethylene analogue, S-benzylcysteine. In order to facilitate further analysis of these results in the light of previous results illustrative experiments with phenylalanine, tyrosine, phenylpyruvic acid, and p-hydroxyphenylpyruvic acid are included. The structural relationships to which reference has been made are indicated in the accompanying chart of compounds, the specific feature under examination being marked in each case with an asterisk.
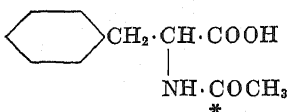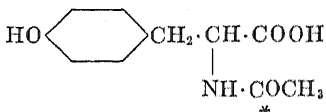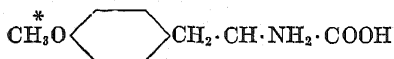
$CH_2 \cdot CH \cdot NH_2 \cdot COOH$

l- or d-phenylalanine

$HO \cdot CH_2 \cdot CH \cdot NH_2 \cdot COOH$

l- or d-tyrosine

$CH_2 \cdot \overset{*}{C}:O \cdot COOH$

Phenylpyruvic acid

$HO \cdot CH_2 \cdot \overset{*}{C}:O \cdot COOH$

p-Hydroxyphenylpyruvic acid

$CH_2 \cdot CH \cdot COOH$
$\underset{*}{NH \cdot COCH_3}$

Acetyl-l-phenylalanine

$HO \cdot CH_2 \cdot CH \cdot COOH$
$\underset{*}{NH \cdot COCH_3}$

Acetyl-l-tyrosine

$\overset{*}{CH_3}O \cdot CH_2 \cdot CH \cdot NH_2 \cdot COOH$

l-Methoxyphenylalanine

$CH_3 \overset{*}{C}O \cdot O \cdot CH_2 \cdot CH \cdot COOH$
$NH \cdot COCH_3$

Diacetyl-l-tyrosine

$\overset{*}{CH_2} \cdot CH_2 \cdot CH \cdot NH \cdot COOH$

dl-Phenylaminobutyric acid

$\overset{*}{CH_2} \cdot S \cdot CH_2 \cdot CH \cdot NH_2 \cdot COOH$
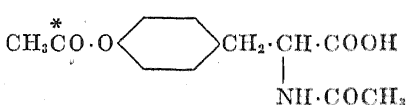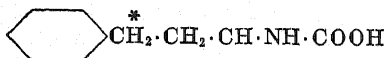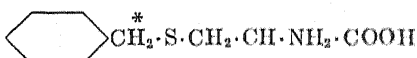
l-S-Benzylcysteine

EXPERIMENTAL

In this study male guinea pigs weighing 300 to 500 gm. were used. They were fed a basal diet of ground Purina rabbit chow (complete ration) which had been exposed to the air in thin layers and thus rendered almost entirely free of vitamin C. The compounds under investigation were administered as a 10 per cent mixture in the basal diet, layered over additional basal diet so that each day's supplement would be entirely consumed. Quantities of these 10 per cent mixtures to furnish 2.76 mM were used so that the amount of compound fed was equivalent to 0.5 gm. of tyrosine, unless otherwise indicated. Vitamin requirements (exclusive of ascorbic acid) were assured

by weekly administration, *per os*, of 0.5 ml. of cod liver oil and daily addition of 0.9 gm. of dried brewers' yeast scattered over the food.

The daily urine samples were collected in glass jars containing 2 N hydrochloric acid and were analyzed immediately after filtration and dilution. The methods of analysis used were those previously employed (2) with the exception that the photoelectric colorimeter was used.

TABLE I

Summary of Urinary Values

Each value represents the daily average for the period indicated. The yield of excreted metabolite is calculated on the basis of the amount of compound fed. The tyrosyl value is determined with tyrosine as the standard and is calculated as mg. of tyrosine.

Compound	Daily amount	No. of days	Ascorbic acid	Excretion values			
				Keto acids	Yield	Tyrosyl value	Yield
	gm.		*mg.*	*mg.*	*per cent of theory*	*mg.*	*per cent of theory*
l-Phenylalanine	0.467	4		164	32.2	196	38.3
	0.953	2	10	11	1.0	0	0
	0.814	2		141	15.9	156	17.5
l-Tyrosine	0.5	4		110	22.0	180	36.1
	0.5	1	20	60.0	12.0	100	20.0
	0.5	2	20	4.7	0.9	31.3	6.3
	0.5	2		67.8	13.6	113	22.6
d-Tyrosine	0.5	7		50.4	10.1	105	21.0
	0.5	5	20	47.9	9.6	135	27.0
"	0.5	7		37.6	7.5	120	24.0
	0.5	5	20	26.9	5.4	114	22.8
"	0.5	3		55.8	11.2	162	32.4
	0.5	3	10	33.3	6.7	166	33.2
	0.5	3	20	17.1	3.4	126	25.2
" with 0.232 gm.	0.5	3		43.7	8.7	138	27.6
NaHCO₃	0.5	3	10	30.3	6.1	141	28.2
	0.5	3	20	22.7	4.5	133	26.6
d-Phenylalanine	0.456	5		45.3	10.0	21.3	4.2
	0.456	4	20	44.2	9.8	21.8	4.4
"	0.456	5		54.4	12.0	23.3	4.7
	0.456	4	20	49.0	10.7	23.8	4.8

The compounds fed were prepared by standard procedures and were extensively purified in order to remove as completely as possible impurities which might interfere with their consumption. *d*-Phenylalanine was obtained by the resolution of the synthetic *dl*-phenylalanine by the method of Fischer and Schoeller (5) as modified by du Vigneaud and Meyer (6). *d*-Tyrosine was prepared by the resolution of the racemic acetyl derivative

(7). Reference to the preparation of the other acetyl derivatives may be found in the same paper (7). The method of Behr and Clarke (8) applied to the crystalline acetyl-*l*-tyrosine yielded *l*-methoxyphenylalanine. The α-keto acids were prepared by the method of Herbst and Shemin (9) and benzylcysteine by the liquid ammonia procedure of du Vigneaud, Audrieth, and Loring (10). Phenylaminobutyric acid was kindly supplied by Professor du Vigneaud and was fed as the racemic compound.

The positive action of ascorbic acid in controlling the metabolism of tyrosine and phenylalanine is indicated in Table I. These illustrative experiments, as previously recorded, show the appearance of metabolites in the urine in the scorbutic state and the prompt disappearance from the urine when ascorbic acid is administered. These two features of the complete picture should be kept in mind in considering the results obtained with the feeding of related compounds.

In contrast, when *d*-tyrosine is fed to the scorbutic animal, approximately 10 per cent appears in the urine as the corresponding α-keto acid and somewhat more as the phenolic compound (tyrosyl value). Of greater importance is the fact, however, that ascorbic acid produces no alteration of these relatively small values even though given in daily quantities of 20 mg. These two facts together must be interpreted as signifying that the vitamin exerts its effect only in the metabolism of the natural isomer. Or in other words, with the opposite configuration of the α-carbon, the structure of the compound is such that it is metabolically handled without regard to the state of vitamin C nutrition.

The significance of the levo configuration is emphasized by the entirely similar results obtained when the unnatural *d*-phenylalanine is fed. Again, 10 per cent appears as keto acid value in the scorbutic state and almost none (only 4 per cent) is excreted as tyrosyl value. Just as in the case of the *d*-tyrosine experiments, the administration of ascorbic acid produces no change in the daily excretion of metabolic intermediates. These points are demonstrated in Table I. Consequently, one must conclude, on the basis of the results obtained by this experimental method, that vitamin C is primarily concerned in metabolic reactions involving the naturally occurring forms of these two amino acids.

That the vitamin C action is even more limited is indicated by the results obtained when the compounds listed in Table II are fed. Whereas phenylpyruvic acid metabolism is vitamin C-dependent, *p*-hydroxyphenylpyruvic acid, as far as urinary analysis is able to show, is metabolized by the scorbutic animal with very little excretion of intermediate products. One experiment with each compound is included from a previous report (2) for purpose of comparison.

Likewise, when the amino group is acetylated, the molecule is no longer

TABLE II

Summary of Analytical Results

Each value represents the daily average for the period indicated. The yield of excreted metabolite is calculated on the basis of the amount of compound fed. The tyrosyl value is determined with tyrosine as the standard and is calculated as mg. of tyrosine.

Compound	Daily amount	No. of days	Ascorbic acid	Excretion values			
				Keto acids	Yield	Tyrosyl value	Yield
	gm.		mg.	mg.	per cent of theory	mg.	per cent of theory
Phenylpyruvic acid	0.694	7		185	24.3	128	16.7
	0.795	5	10	101	12.7	0	0
	0.831	6		146	16.0	79	8.6
Hydroxyphenylpyruvic acid	1.00	5		33.8	3.4	151	15.1
	1.06	2	10	55.4	5.2	176	16.6
	1.44	2		55.5	3.9	237	16.5
N-Acetyl-*l*-phenylalanine	0.572	5		8.5	1.9	22.8	4.6
	0.572	4	25	7.7	1.7	24.4	4.9
N-Acetyl-*l*-tyrosine	1.04	9		2.1	0.3	123.0	14.6
	0.97	5	10	2.6	0.3	152.0	19.3
"	0.616	7		5.3	1.1	49.8	10.0
	0.616	5	20	6.1	1.2	74.2	14.8
"	0.304	7		5.4	2.1	48.8	19.8
	0.365	3	20	5.1	1.7	52.6	17.8
"	1.00	8		4.4	0.5	110	13.5
	1.17	3	20	4.9	0.5	134	14.1
"	0.717	8		2.3	0.4	65.7	11.3
	0.930	5	10	3.4	0.5	93.8	12.4
" with	0.616	7		5.8	1.2	44.7	8.9
0.232 gm. NaHCO₃	0.616	5	20	7.3	1.5	72.7	14.5
Diacetyl-*l*-tyrosine	0.732	5		5.1	0.7	80.2	11.7
	0.732	4	25	4.8	0.7	76.8	11.2
l-p-Methoxyphenyl- alanine	0.539	4		129	28.5	26.4	5.3
"	0.539	4	20	88.5	19.5	21.3	4.3
	0.467	4		79.4	17.0	16.5	3.8
"	0.356	5	20	76.2	23.1	15.6	4.7
	0.500	4		65.9	15.6	27.2	5.9
	0.500	5	20	47.8	11.4	35.5	7.6
	0.500	2		36.3	8.6	32.8	7.1
"	0.505	6		91.3	21.5	21.8	4.7
	0.539	5	20	136	30.0	21.8	4.3
dl-Phenylaminobutyric acid	0.500	2		153	32.2	0	0
	0.500	6	18	132	28.7	0	0
l-S-Benzylcysteine	0.473	3		74.3	22.0	0	0
	0.500	3	20	77.0	19.8	0	0

under the control of vitamin C, as the values in Table II show. It must be pointed out that one could have expected the acetyl group to be removed by hydrolysis in the intestine. Had this occurred, however, the results would have been essentially the same as those obtained in the feeding of the free amino acid. The results are distinctly different; therefore hydrolysis must not have occurred and thus the results obtained clearly demonstrate the importance of the free amino group in the course of the metabolic processes controlled by the vitamin. The excretion values with both acetyl-*l*-tyrosine and acetyl-*l*-phenylalanine are extremely low in both the vitamin C-free and vitamin C-supplemented periods, as may be seen in Table II.

The inherent structure of tyrosine permits further evaluation of essential structural features, for the phenolic hydroxyl group may also be acetylated. As shown in Table II, the feeding of diacetyl-*l*-tyrosine led to entirely negative results as far as dependence upon the vitamin is concerned. The results summarized in Table II do not permit complete distinction between the compound and N-acetyl-*l*-tyrosine, for hydrolytic cleavage of the O-acetyl group cannot be entirely eliminated. The same question, however, may be examined by the use of a compound in which the para position is substituted with a group believed to be considerably more stable under physiological conditions. The feeding of *l*-*p*-methoxyphenylalanine (Table II) resulted in the excretion of around 20 per cent of the fed compound as α-keto acid but the administration of ascorbic acid did not affect the amount excreted. The tyrosyl values at the same time were very low (basal values) in both periods. Consequently it must be concluded that the introduction of the methyl group alters the original amino acid structure sufficiently so that it no longer participates in the series of reactions regulated by the vitamin.

It should be pointed out that in these experiments the *p*-methoxyphenyl-pyruvic acid proved to be so insoluble that it crystallized from the urine. By rinsing the collecting funnel and jar with alcohol it was returned to solution for purpose of analysis. The 20 per cent referred to above and in Table II therefore includes the total amount excreted. The marked insolubility of the compound made it possible to isolate, purify, and identify it by chemical means.

Further modification of the amino acid molecule is made possible by lengthening the side chain and the interpolation of a thiomethylene group in the side chain, as represented in the compounds phenylaminobutyric acid and *l*-S-benzylcysteine, respectively. In each case the conversion of the compound to keto acid which amounted to 15 to 30 per cent was unaffected by administration of vitamin C to the scorbutic guinea pig. Examination of Table II also shows that no significant production of phenolic compound occurred.

DISCUSSION

By means of urinary analysis, results have been obtained which clearly point to a very selective action of ascorbic acid in connection with the metabolism of phenylalanine and tyrosine. Only these two amino acids and phenylpyruvic acid have shown any dependence upon the intake of ascorbic acid. All of the others which represent various modifications of the original amino acid structures proved to be completely independent of the state of vitamin C nutrition. Additional emphasis to this conclusion is available in a consideration of the analytical methods employed. The three methods chiefly used permit detection of alterations in the urinary content of ketone compounds (2,4-dinitrophenylhydrazine), phenolic substances (Bernhardt-Millon), and reducing molecules (acid phosphomolybdate). In addition extensive fractionations have been utilized in an effort to find compounds which might have escaped the reactivity of the analytical reagents.

The modification of structure, a single feature at a time, which produced metabolically vitamin-independent compounds included each of the more obvious component parts of tyrosine. Represented were the levo configuration of the α-carbon, the unsubstituted amine radical, the unsubstituted para position in the benzene nucleus, and the characteristic alanine side chain. In addition, the α-keto acid which is produced by the oxidative deamination of tyrosine exhibited no dependence upon ascorbic acid in the intact guinea pig.

The results obtained with the phenylalanine and tyrosine derivatives may be regarded, in one light, as being negative. They have, however, guided the problem into a more direct attack; namely, the study of specific tissues and their ability to oxidize tyrosine when supplied with adequate and inadequate quantities of the vitamin in question. The results so far obtained (11) and the subsequent application of the *in vitro* method already promise a more complete understanding of the findings described in this instance.

SUMMARY

A series of phenylalanine and tyrosine derivatives has been fed to scorbutic guinea pigs with and without ascorbic acid supplementation.

The compounds used were d-tyrosine, d-phenylalanine, N-acetyl-l-phenylalanine, N-acetyl-l-tyrosine, diacetyl-l-tyrosine, l-p-methoxyphenylalanine. dl-phenylaminobutyric acid, and l-S-benzylcysteine. Each represents a specific modification of the original amino acid structure.

The values reported for daily urinary excretion of keto acid and phenolic (tyrosyl) values for each compound have exhibited no correlation with the state of ascorbic acid nutrition. With this method of study it then may be

concluded that each is metabolized by the guinea pig independently of ascorbic acid.

Added emphasis has thus been given to the previously recorded finding that *l*-phenylalanine, phenylpyruvic acid, and *l*-tyrosine are the compounds exhibiting an ascorbic acid-dependent metabolism.

BIBLIOGRAPHY

1. Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135**, 251 (1940).
2. Sealock, R. R., Perkinson, J. D., Jr., and Basinski, D. H., *J. Biol. Chem.*, **140**, 153 (1941).
3. Levine, S. Z., Marples, E., and Gordon, H. H., *J. Clin. Invest.*, **20**, 199 (1941).
4. Levine, S. Z., Gordon, H. H., and Marples, E., *J. Clin. Invest.*, **20**, 209 (1941).
5. Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 1 (1907).
6. du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98**, 143 (1932–33).
7. Sealock, R. R., *J. Biol. Chem.*, **166**, 1 (1946).
8. Behr, L. D., and Clarke, H. T., *J. Am. Chem. Soc.*, **54**, 1630 (1932).
9. Herbst, R. M., and Shemin, D., in Organic syntheses, New York, **19**, 77 (1939).
10. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52**, 4500 (1930).
11. Lan, T. H., and Sealock, R. R., *J. Biol. Chem.*, **155**, 483 (1944).

REMOVAL OF PLASMA PHOSPHOLIPIDES AS A FUNCTION OF THE LIVER: THE EFFECT OF EXCLUSION OF THE LIVER ON THE TURNOVER RATE OF PLASMA PHOSPHOLIPIDES AS MEASURED WITH RADIOACTIVE PHOSPHORUS

By C. ENTENMAN, I. L. CHAIKOFF, AND D. B. ZILVERSMIT

(From the Division of Physiology, University of California Medical School, Berkeley)

Although the reaction involving the incorporation of inorganic phosphate into phospholipides is not restricted to any one tissue (surviving slices of liver, kidney, brain, spinal cord, small intestine, and muscle (1) have been shown to effect this reaction), the phospholipides contained in plasma are synthesized mainly by the liver (2). This function of the liver in the formation of plasma phospholipides was demonstrated by comparing the rates at which labeled inorganic phosphate introduced into the blood stream is converted to labeled plasma phospholipides by the normal and the liverless dogs; negligible amounts of labeled phospholipides were recovered in the plasma of the latter as late as 6 hours after excision of the liver (2).

The present investigation deals with another phase of the metabolism of plasma phospholipides; namely, their removal as measured by the rate at which intravenously introduced, labeled (such as radioactive) phospholipides disappear from the blood stream.

EXPERIMENTAL

Preparation of Labeled Plasma Phospholipides—The method used here for obtaining radioactive plasma phospholipides has been described elsewhere (3). Each of the donor dogs received by stomach tube 2 to 3 millicuries of radioactive inorganic phosphate. Blood was removed from the dogs 20 to 30 hours later, treated with heparin, and the plasma separated by centrifugation. This plasma, which contained the radioactive phospholipides, was injected intravenously into dogs and the rate of disappearance of the labeled phospholipides from their plasma measured.

Preparation of Animals—The dogs used in this study were maintained for about 3 weeks on a stock diet, the composition of which has been described elsewhere (4). After being fasted for 24 hours they were eviscerated in the following manner: Each dog was anesthetized with ether and injected with 1 mg. of atropine sulfate to inhibit salivation. The abdominal cavity was opened and the inferior mesenteric artery ligated and severed. Two clamps were applied to the lower end of the pelvic colon and the gut severed between them. The celiac axis was exposed, its arteries clamped, and their

connections with the gastrointestinal tract severed. The portal vein was cut in a similar manner. The esophagus was clamped just below the diaphragm and the entire gastrointestinal tract removed from the abdominal cavity, but the liver was left *in situ*. A towel wet with saline was placed in the cavity and the abdominal cavity closed. The entire procedure as a rule occupied less than 15 minutes. After recovery from the anesthetic the dogs behaved normally and were in no apparent pain. Blood was removed for analyses at various intervals throughout the experiment.

It should be noted that the eviscerated animals showed no symptoms of shock. They walked around and behaved in a normal manner. Hematocrit readings, obtained each time that blood samples were taken, did not deviate from the normal. Such observations are in agreement with those of other investigators (5).

Analytical Procedures—The methods employed for determination of phospholipide P^{31} and phospholipide P^{32} have been described elsewhere (3). Total fatty acids and cholesterol were determined by oxidative methods (6).

Results

The liver of the eviscerated dog, although it received no blood by way of the portal vein or hepatic artery, is left *in situ*. Several investigators have pointed out that in such a preparation the liver is not completely excluded from the circulation (7). In order to test the *functional* significance of the residual circulatory connections of the liver of the eviscerated dog, its capacity to contribute phospholipides to plasma was investigated.

Test of Evisceration—Eight dogs were eviscerated and immediately thereafter injected intravenously with radioactive inorganic phosphate. Each dog received 10 cc. of an isotonic solution of Na_2HPO_4 containing 2 millicuries of radioactive phosphorus. Blood was removed from the animals at intervals of 4 to 9 hours after the radiophosphorus had been introduced into the circulation, and radiophospholipides of the plasma determined. It is apparent from Table I that the quantity of radiophospholipides in the plasma of the eviscerated dog is negligible as compared with that previously observed in normal dogs (8). Since plasma phospholipides are synthesized in the liver, it may be concluded that any circulation of the liver remaining after the portal vein and hepatic artery are severed is of little significance so far as the synthesis of plasma phospholipides is concerned.

Effect of Evisceration on Lipide Content of Plasma—The effect of evisceration upon the concentration of phospholipides, total fatty acids, and cholesterol in plasma is recorded in Table I. In each dog the level of each lipide constituent was measured immediately before and at one or more intervals after evisceration.

No appreciable decrease in the concentration of phospholipides occurred in any of the eight dogs examined. The maximum difference between the values found before and after evisceration did not exceed 12 per cent.

The levels of total fatty acids in the plasma were also unaffected by evisceration. In seven of the eight dogs the values for total fatty acids

TABLE I

Plasma Lipides of Eviscerated Dog (with Hepatic Artery Severed)

The lipides are expressed as mg. per 100 cc. of plasma.

Dog No.	Body weight	Hrs. after evisceration	Cholesterol			Total fatty acids	Total lipides	Phospho- lipide	Specific activity of PLP,* $\times 10^3$
			Total	Free	Ester				
	kg.								
EV3†	12.5	Before	206	72	134	535	741	385	
		4.5	204	81	123	542	746	368	
		9	202	77	125	580	782	388	0.20
EV5†	8.5	Before	173	80	93	515	688	334	
		5	152	80	72	535	687	314	
		7.5	165	81	84	535	700	319	0.45
EV6	8.8	Before	271	67	204	440	711	375	
		4.5	262	63	199	480	742	345	
		8	223	67	156	525	748	363	0.35
EV7	11.7	Before	115	38	77	306	421	206	
		3.5	113	53	60	440	553	187	
		4.75	103	46	57	295	398	180	0.21
EV10†	8.0	Before	188	63	125	417	605	281	
		10	196	63	133	397	593	253	0.13
3-46	18.2	Before	108	48	60	298	406	172	
		5	93	25	68	324	417	158	0.18
3-50A	10.0	Before	154	45	109	424	578	270	
		5	146	44	102	427	573	250	0.16
3-50B	9.0	Before	174	65	109	408	582	300	
		4	169	81	88	354	523	284	0.17

* PLP refers to phospholipide phosphorus, r. u. to radioactive units as determined by the Geiger-Müller counter. Specific activity is calculated as r. u. PLP^{32}:mg. PLP^{31}, the PLP^{32} being expressed as a percentage of the injected P^{32}.

† Received intravenously 2 gm. of glucose in 25 cc. of isotonic saline every hour following evisceration.

observed after evisceration were either the same as, or slightly higher than, the corresponding preoperative values (Table I). The concentration of total fatty acids found in the plasma of Dog 3-50B 4 hours after evisceration is about 15 per cent lower than the preoperative value.

No demonstrable change in the level of either free or ester cholesterol was produced by evisceration.

TABLE II

Disappearance of Injected Radiophospholipides from Plasma of Eviscerated Dogs (with Hepatic Artery Severed)

Dog No.	Body weight	Mins. after PLP^{32}* injection	R. u.† PLP^{32} per cc. plasma‡	Mg. PLP^{31} per cc. plasma	Specific activity of PLP, $\times 10^{-2}$	Turnover time of plasma phospholipide before or after evisceration (min.)‡
	kg.					
1	9.0	(Eviscerated)				
		45	795	0.122	6.50	
		180	597	0.106	5.62	
		300	521	0.104	5.00	
		360	475	0.108	4.40	After 920
2	8.7	55	1156	0.073	15.8	
		60 (Eviscerated)				
		90	1140	0.073	15.7	
		185	1028	0.073	14.1	
		270	1030	0.073	14.1	
		345	1000	0.071	14.1	After 10,000
3	6.8	55	1540	0.167	9.21	
		60 (Eviscerated)				
		75	1540	0.173	8.90	
		175	1270	0.176	7.22	
		255	1188	0.173	6.85	
		330	1010	0.176	5.74	After 640
4	6.5	90	584	0.117	5.00	
		100 (Eviscerated)				
		110	577	0.117	4.92	
		170	562	0.116	4.85	
		230	537	0.116	4.65	
		290	527	0.115	4.58	After 2500
5	7.8	95	592	0.110	5.40	
		105 (Eviscerated)				
		115	585	0.107	5.47	
		175	547	0.105	5.22	
		250	530	0.105	5.07	After 2000
6	8.7	40	517	0.074	7.03	Before 380
		95	367	0.062	5.95	
		180	347	0.072	4.86	
		185 (Eviscerated)				
		210	380	0.078	4.44	
		255	348	0.078	4.35	
		315	273	0.067	4.12	
		375	268	0.065	4.18	After 2250

TABLE II—*Concluded*

Dog No.	Body weight	Mins. after PLP^{32}* injection	R.u.† PLP^{32} per cc. plasma‡	Mg. PLP^{31} per cc. plasma	Specific activity of PLP, ×10^{-3}	Turnover time of plasma phospholipide before or after evisceration (min.)‡
	kg.					
7	15.0	60	1080	0.131	8.78	Before 420
		125	940	0.123	7.64	
		210	775	0.123	6.30	
		215 (Eviscerated)				
		265	733	0.119	6.16	
		305	686	0.112	6.12	
		370	667	0.112	5.95	
		430	665	0.113	5.89	After 3400
8	18.0	50	870	0.120	7.25	Before 410
		145	734	0.126	5.82	
		215	630	0.131	4.81	
		220 (Eviscerated)				
		275	600	0.123	4.87	
		340	585	0.123	4.75	
		415	540	0.125	4.31	After 4750
9	7.0	60	2575	0.124	20.8	Before 580
		120	2180	0.126	17.2	
		180	2100	0.128	16.4	
		185 (Eviscerated)				
		265	2090	0.128	16.3	
		325	2050	0.127	16.2	
		385	2030	0.127	16.0	After 9500

* PLP refers to phospholipide phosphorus.

† Radioactive units as determined by the Geiger-Müller counter. The specific activity is calculated as the ratio of r. u. PLP^{32}:mg. PLP^{31}.

‡ The turnover time is defined as the time required for the disappearance of an amount of plasma phospholipide equal to that present in the plasma (3).

Phospholipide Turnover Time—Since in the absence of the liver labeled plasma phospholipides were not synthesized from injected P^{32}, the finding that the level of this plasma lipide remained constant in the liverless dog suggests that in the latter the removal of plasma phospholipides had practically ceased. To test this point, evisceration and severance of the hepatic artery were carried out in nine dogs (Table II). Before evisceration all nine dogs received intravenously plasma containing radioactive phospholipide. The changes that occurred in phospholipide P^{32} and phospholipide P^{31} with time are recorded in Table II. The *turnover time* for plasma phospholipide, *i.e.* the time required for the disappearance of an amount of plasma phospholipide equal to that present in the plasma, was

calculated after the manner described elsewhere (3), and the values shown in Table II.

In the first five dogs (Table II) the radiophospholipide was injected *just before* evisceration; it was therefore possible to obtain measures of turnover

TABLE III

Disappearance of Injected Radiophospholipides from Plasma of Eviscerated Dogs (with Hepatic Artery Intact)

Dog No.	Body weight	Mins. after PLP^{32} injection*	R. u.† PLP^{32} per cc. plasma	Mg. PLP^{31} per cc. plasma	Specific activity of PLP,‡ ×10^{-2}	Turnover time of plasma phospholipide before and after evisceration (min.)
	kg.					
10	19.0	45	690	0.102	6.76	Before 280
		75	620	0.103	6.08	
		107	585	0.101	5.73	
		130 (Eviscerated)				
		135	604		4.54	
		195	502		3.78	
		263	416		3.12	
		315	409	0.133	3.07	After 280
11	12.5	43	713	0.133	5.48	Before 430
		74	705		5.41	
		105	632	0.127	4.85	
		135 (Eviscerated)				
		140	651	0.140	4.65	
		205	557	0.140	4.29	
		260	444	0.140	3.17	
		335	404	0.145	2.88	After 430
12	15.0	42	822	0.100	7.98	Before 350
		75	746	0.103	7.25	
		135	630	0.104	6.11	
		150 (Eviscerated)				
		157	630	0.107	6.00	
		217	490		4.66	
		280	402	0.106	3.82	
		337	364	0.103	3.47	After 350

* PLP refers to phospholipide phosphorus.

† Radioactive units as determined by the Geiger-Müller counter.

‡ Specific activity is calculated as the ratio of r. u. PLP^{32}:mg. PLP^{31}.

times only for the interval *after* evisceration. The values obtained, even though much higher than normal, varied considerably. This variability made it desirable to compare the turnover time of plasma phospholipides in the same dog before and after evisceration. This was done in the last four dogs in Table II. The values found for turnover time in these four

dogs before evisceration fell within the range reported earlier (8); they were respectively 380, 420, 410, and 580 minutes. Following evisceration the values increased to 2250, 3400, 4750, and 9500 minutes, respectively.

It is evident from the above data that in the eviscerated dog the turnover of plasma phospholipides practically stops. In this type of preparation, the entire gastrointestinal tract is excised and the liver excluded from the circulation. In order to determine whether this impaired turnover results from the removal of the liver or from the removal of the gastrointestinal
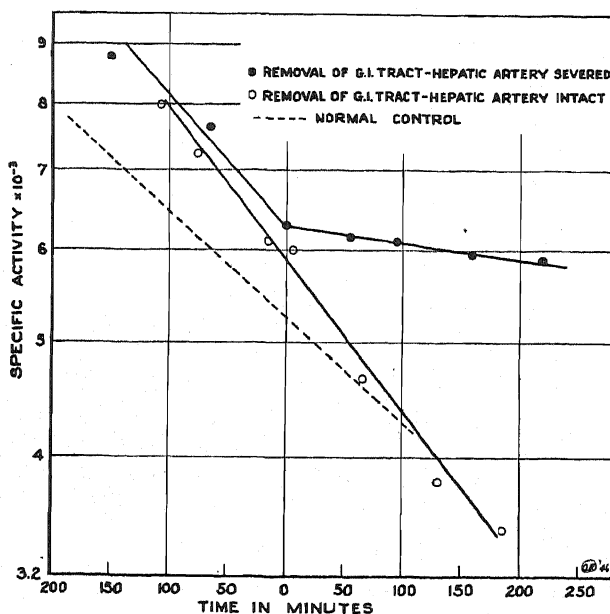
FIG. 1. Semilogarithmic plot of the specific activities of phospholipide phosphorus against time. ● = Dog 7 (Table II); O = Dog 10 (Table III); – – – – = the average of three normal dogs (3).

tract or from both, the following experiment was carried out: Three dogs were injected with plasma containing radioactive phospholipides, and 130 to 150 minutes later were eviscerated in the manner previously described, *with the exception that the blood supply to the liver via the hepatic artery was not interfered with*. The changes in the phospholipide P^{32} and phospholipide P^{31} content of plasma in these dogs and the turnover times are presented in Table III. The data demonstrate clearly that, if the arterial blood supply to the liver is intact, the complete removal of the gastrointestinal tract, pancreas, spleen, etc., has no effect on the rate of disappearance of plasma phospholipides.

Fig. 1 shows a plot of the specific activities of plasma phospholipide

phosphorus on a logarithmic scale against time. The curves were drawn from data obtained for (1) the average of three normal dogs (3); (2) an eviscerated dog (Dog 7, Table II) in which the liver was deprived of blood supply from portal vein and hepatic artery; (3) an eviscerated dog (Dog 10, Table III) in which the blood supply to the liver via the hepatic artery was left intact. The disappearance of labeled phospholipides from the plasma of the normal dog proceeds at a constant rate throughout the 5 hour experiment. So long as the blood supply to the liver by way of the hepatic artery is left intact, removal of the gastrointestinal tract has no apparent effect on the disappearance of labeled phospholipide from the plasma. *When, however, the blood supply to the liver via the hepatic artery was eliminated in the eviscerated dog, there occurred an abrupt decrease in the rate at which labeled phospholipides disappeared from the plasma.*

DISCUSSION

6 hours after the injection of radioactive inorganic phosphate, negligible amounts of radiophospholipide can be recovered from liverless dogs made so either by complete excision of the liver (2) or, as in the present study, by excluding this organ from the circulation. Although such findings have been offered as evidence for the view that the liver is the main site for synthesis of plasma phospholipide, the possibility that the extrahepatic tissues contribute to plasma appreciable amounts of phospholipide of low specific activity was not ruled out. That this does not occur, however, is shown by the data contained in Column 6 of Table II and by Fig. 1. The specific activities of plasma phospholipide phosphorus of dogs that had received intravenously radioactive plasma phospholipides did not decrease significantly after the liver had been excluded from the circulation. If, in the eviscerated dog with the liver excluded from the circulation, new plasma phospholipide had been formed, the specific activities of plasma phospholipide phosphorus could not have remained so constant.

Since plasma phospholipides are not synthesized in the absence of the liver, the finding that the level of plasma phospholipides remains constant or nearly so in the liverless dog means that in the latter the utilization of plasma phospholipides has practically ceased. This suggests that the liver is also the main tissue in which their utilization occurs. Further evidence for this concept is provided by a comparison of the rates at which the injected labeled plasma phospholipides disappeared from the plasma of the normal and the liverless dogs. The fact that these labeled plasma phospholipides disappeared at a normal rate when the gastrointestinal tract was removed but not when the liver was excluded can leave little doubt as to the significance of the liver in the utilization of plasma phospholipides.

The turnover of plasma phospholipides did not completely stop in all

of the eviscerated dogs.   This may be due to small amounts of blood reaching the liver by routes other than the portal vein and hepatic artery.   On the other hand, the small decrease in labeled plasma phospholipide in the eviscerated dogs may be indicative of some utilization of plasma phospholipide by extrahepatic tissues, a utilization, however, that is negligible as compared with that observed in dogs in which the liver remains intact.

The results presented here raise the interesting question as to what function plasma phospholipides serve in the animal body.   The finding that the liver alone is so much more important than the total mass of extrahepatic tissues in their utilization lends little support to the view that the main function of plasma phospholipides is to transport fatty acids from liver to other tissues.

SUMMARY

1. The concentration of phospholipides, total fatty acids, and cholesterol in plasma is not appreciably decreased by excluding the liver from the circulation.

2. The rate at which intravenously injected radiophospholipide disappears from the plasma was used as a measure of the turnover time of plasma phospholipides.   In confirmation of earlier work, it was found that plasma phospholipides are completely turned over in 6 to 10 hours in normal dogs weighing from 7 to 18 kilos.   By depriving these dogs of their livers, the time required for complete turnover was prolonged to 33 to 160 hours.

3. It is concluded that the liver is the principal tissue in the body concerned not only with the synthesis and supply of plasma phospholipides but also with their *removal*.

BIBLIOGRAPHY

1. Chaikoff, I. L., *Physiol. Rev.*, **22**, 291 (1942).
2. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **150**, 47 (1943).
3. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333 (1943).
4. Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **142**, 129 (1942).
5. Markowitz, J., Text-book of experimental surgery, Baltimore, 493 (1937).
6. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **106**, 267 (1934).
7. Peterson, J. M., *Physiol. Rev.*, **14**, 586 (1934).
8. Friedlander, H. D., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **158**, 231 (1945).

ADRENALIN IN SCURVY

By SACHCHIDANANDA BANERJEE AND NARESH CHANDRA GHOSH

(From the Department of Mitra Research in Diabetes, Calcutta School of Tropical Medicine, and the Applied Chemistry Department, University College of Science and Technology, Calcutta, India)

It was reported earlier (1) that there was a significant increase in the adrenalin content of the adrenal glands of scorbutic guinea pigs. Unfortunately the paired feeding technique was not then employed. As inanition is always associated with the onset and development of scurvy, it might be argued that the results obtained were due to inanition alone and not to the specific effect of vitamin C deficiency. In the present investigation, therefore, an attempt was made to study the relation of scurvy to the adrenalin content of the adrenal glands of guinea pigs by the use of the paired feeding technique.

We observed a diabetic type of glucose tolerance curve in scorbutic guinea pigs (2) and pointed out that the lowered glucose tolerance of the scorbutic guinea pigs compared with that of normal animals might be partly due to an increased action of adrenalin (1) in the absence of the opposing action of insulin (3). The rôle of the adrenal medulla on the glucose tolerance test was, therefore, studied in guinea pigs in which the medulla had been removed; a scorbutic diet with or without the supplement of ascorbic acid and the paired feeding technique were employed.

EXPERIMENTAL

Relation of Scurvy to Adrenalin Content of Adrenal Glands of Guinea Pigs

Young male guinea pigs with an average weight of 225 gm. were fed *ad libitum*, for 1 week, a scorbutic diet (1) with a daily supplement of 5 mg. of ascorbic acid. Those animals which were growing on this diet were selected. They were divided into two groups, housed in individual cages, and paired evenly so that the weights of the animals in each pair, one from each of the groups, were about the same. One of the groups was fed *ad libitum* the scorbutic diet without any supplement of ascorbic acid for 20 days. The daily food consumption of each animal of this group was measured and an equivalent amount of the scorbutic diet was given to the corresponding animal of the second group which received in addition a daily oral supplement of 5 mg. of ascorbic acid. All the animals were fed 2 drops of a concentrate of vitamins A and D twice a week. In the evening of the 20th day the food was removed from the cages of all the animals. The next morning, the animals were sacrificed by a blow on the

head, the neck veins cut, the adrenals removed and transferred to a weighed tube containing normal saline and a few drops of 10 per cent trichloroacetic acid, and the whole weighed again. Adrenalin was determined by the method previously described (1). The results are shown in Table I.

TABLE I

Determination of Adrenalin in Normal and Scorbutic Guinea Pigs

Pair No.	Weight at death		Weight of adrenals		Weight of adrenals per 100 gm. body weight		Adrenalin per 1 gm. adrenal	
	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
	gm.	gm.	mg.	mg.	mg.	mg.	γ	γ
1	308	286	132.6	153.2	43	54	233	515
2	250	220	Lost	106.8		49		731
3	270	252	111.6	115.8	41	46	202	604
4	260	220	137.6	186.8	53	85	210	437
5	238	230	121.4	161.8	51	70	186	449
6	258	242	111.8	146.2	43	60	238	510
7	238	215	123.0	124.8	52	58	234	577
8	296	258	134.4	216.0	45	84	169	371
9	335	240	136.0	159.0	41	66	220	478
10	227	189	82.4	106.4	36	56	207	670
11	228	186	126.6	139.4	55	75	173	503
12	304	292	167.8	182.6	55	62	120	447
13	266	226	167.4	190.0	63	84	137	418
14	297	265	143.2	207.6	48	78	144	344
15	165	155	75.4	118.6	46	77	286	444
16	330	272	168.6	193.2	51	71	123	433
17	240	209	111.2	155.6	46	74	258	427
18	225	211	103.2	170.2	46	81	135	335
Average..................			127.2	157.4	48	68	198	483
Difference of means...........................					20		285	
Standard error of difference...................					3.65		28.01	
t*...					5.48		10.17	

* All values of *t* are highly significant.

Relation of Adrenal Medulla to Glucose Tolerance Test in Normal and Scorbutic Guinea Pigs

Three groups of guinea pigs, each containing three animals, were housed in separate cages and fed *ad libitum* the scorbutic diet with daily oral supplements of 5 mg. of ascorbic acid. Two animals from each group were selected. They were fasted overnight and on the 6th day adrenal enucleation was performed under ether anesthesia according to the method, slightly modified, described by Evans for rats (4). After the operation,

the animals were allowed to drink a 1 per cent solution of sodium chloride. On the 22nd day after demedullation, by which time the cortex is completely regenerated, the ascorbic acid supplement was withdrawn from one of the demedullated animals in each group. The daily consumption of the diet of the animal receiving no ascorbic acid was measured and an equivalent amount of the diet was given separately to the other two of the group.
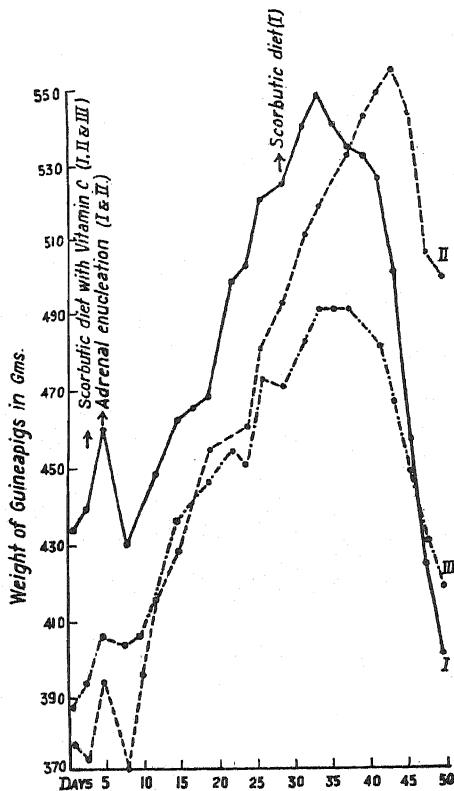
FIG. 1. Growth curves of guinea pigs. Curve I shows the growth of the scorbutic guinea pig with demedullated adrenals; Curve II, the demedullated guinea pig receiving vitamin C; Curve III, the guinea pig with intact adrenals receiving vitamin C.

Thus, in each group, one demedullated animal received the scorbutic diet only, while the other demedullated guinea pig and also the animal with intact adrenals received an equivalent amount of the scorbutic diet with a daily oral supplement of 5 mg. of ascorbic acid. All the animals were fed 2 drops of a concentrate of vitamins A and D twice a week during the experimental period. In the evening of the 49th day of the experiment, 21 days after the withdrawal of ascorbic acid from one of the demedullated

guinea pigs of each group, food was withdrawn from the cages of all the animals. The glucose tolerance test was performed the next morning. After a fasting blood specimen had been taken from the heart, each guinea pig was fed 0.2 gm. of glucose per 100 gm. of body weight in a 50 per cent solution. Blood samples were taken from the heart at intervals of 45 minutes up to 280 minutes after the administration of glucose. The blood sugar was estimated according to the method of Hagedorn and Jensen (5).
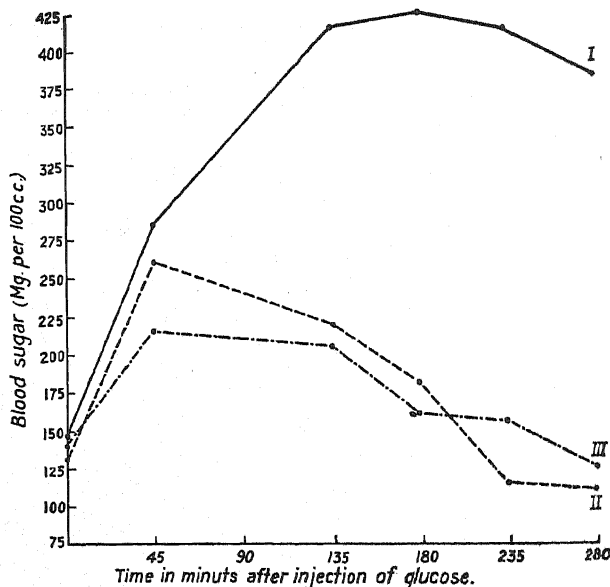
FIG. 2. Glucose tolerance curves of guinea pigs. Curve I represents the results on the scorbutic guinea pig with demedullated adrenals; Curve II, the demedullated guinea pig receiving vitamin C; Curve III, the guinea pig with intact adrenals receiving vitamin C.

Curves for growth and glucose tolerance of one of the groups of guinea pigs are shown in Figs. 1 and 2.

DISCUSSION

Both the size and the adrenalin content of the adrenal glands of scorbutic guinea pigs were significantly increased when compared with those of the pair-fed normal control animals (Table I). In our previous report (1), in which the normal control animals received the diet *ad libitum*, both the size and the adrenalin content of the adrenal glands were relatively higher. Inanition, therefore, slightly affects the above findings. The increase in the size and the adrenalin content of the adrenals in scurvy is therefore mainly due to the effect of vitamin C deficiency. In the present investi-

gation all the scorbutic animals were fed *ad libitum* the scorbutic diet for 20 days, when they were sacrificed. Unlike the scorbutic animals of the previous report (1), which received the scorbutic diet up to 25 days, the animals of the present experiment were in the early stage of scurvy and the size and adrenalin content of the adrenal glands were at a relatively lower level. It seems, therefore, that the size of the adrenal glands and the adrenalin content increase gradually with the progress of scurvy.

Fig. 1 shows that the normal control animals, fed the same amount of diet as the animal of the scorbutic group, lose weight similar to the scorbutic animals, but the effect is delayed. The loss in weight with the progress of scurvy is therefore mainly due to inanition. The demedullated scorbutic guinea pig gave a diabetic type of glucose tolerance curve like that observed in scorbutic guinea pigs (2). The demedullated guinea pig and the control with intact adrenals, which received the same amounts of diet consumed by the scorbutic animal in addition to the supplement of vitamin C, gave normal glucose tolerance curves. This indicates that neither adrenalin nor inanition is responsible for the lowered glucose tolerance as observed in scurvy. The disturbed carbohydrate metabolism in scurvy seems more likely to be due to diminished insulin secretion by the pancreas of the scorbutic guinea pigs (3).

SUMMARY

1. The effect of scurvy on the adrenalin content of the adrenals of guinea pigs was studied by a paired feeding technique.

2. There was significant increase in the size and also in the adrenalin content of the adrenals of scorbutic guinea pigs.

3. The glucose tolerance tests were performed on scorbutic guinea pigs with demedullated adrenals, on demedullated guinea pigs receiving vitamin C, and on guinea pigs with intact adrenals receiving vitamin C.

4. The demedullated scorbutic guinea pigs gave a diabetic type of glucose tolerance curve, whereas the others showed a normal tolerance to glucose.

5. Neither adrenalin nor inanition was responsible for the lowered glucose tolerance observed in scurvy.

BIBLIOGRAPHY

1. Banerjee, S., *J. Biol. Chem.*, **159**, 327 (1945).
2. Banerjee, S., *Ann. Biochem. and Exp. Med.*, **3**, 157 (1943).
3. Banerjee, S., *Nature*, **152**, 329 (1943); *Ann. Biochem. and Exp. Med.*, **4**, 33 (1944).
4. Evans, G., *Am. J. Physiol.*, **114**, 297 (1936).
5. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **137**, 92 (1923).

THE COMPONENT FATTY ACIDS OF BUFFALO COLOSTRUM FAT

By C. P. ANANTAKRISHNAN, V. R. BHALE RAO, T. M. PAUL, AND M. C. RANGASWAMY

(From the Imperial Dairy Research Institute, Bangalore, India)

The fluid secreted immediately after parturition differs in chemical composition from normal milk in many respects. Many studies have been made of the properties and composition of the colostrum of cows, but very little work has been done on the colostrum of other animals. In India, the buffalo is an important source of milk and butter fat, which have been the subject of many investigations. However, no information seems to be available on the composition of the colostrum or the fat of the milk secreted by the animal during the first few days after parturition. A study on this subject might throw some light on the physiology and the mechanism of the fat synthesis and secretion by the mammary glands of the buffalo.

EXPERIMENTAL

The fat was separated from the colostrum and milk of four Murrah buffaloes maintained at the Institute farm. All the animals were milked twice a day and the fluid secreted every 24 hours from each animal was mixed and the butter fat prepared from this mixture. Collection of butter fat was continued up to the 15th day of lactation. Representative composite samples of each day of lactation were obtained and analyzed for the usual chemical characteristics; the results are presented in Table I. The mixtures obtained on the 1st, 2nd, 3rd, and 10th days of lactation were subjected to detailed chemical analysis by ester fractionation according to the method of Hilditch as modified by Smith and Dastur (1); a summary of the results is presented in Table II.

All the animals at about a month before calving were fed daily 3 pounds of a concentrated mixture composed of wheat bran, groundnut cake, Bengal gram (*Cicer arietinum*), and Bengal gram husk, in the ratio of 4.0:2.5:1.5:2.0, in addition to 3 pounds of wheat bran. For the first 4 days after calving the animals received 10 pounds of wheat bran and 1 pound of jaggery (crude cane sugar). Throughout the period the roughage consisted of 70 pounds of mixed green grass and 5 pounds of ragi straw. From the 5th day of lactation onwards the amount of concentrated mixture fed was adjusted according to the milk yield at the rate of 1 pound for every 2 pounds of milk produced by the animal.

The results in Table I indicate that as lactation advances there is a

31

TABLE I

Analytical Characteristics of Fat of Composite Mixtures of Buffalo Colostrum

Day of lactation	M.p.	Butyrometer reading at 40°	Saponi-fication value	Iodine value	Reichert value	Polenske value	Kirschner value
	°C.						
1	39.1	44.5	218.7	38.6	25.08	0.90	20.82
2	36.8	43.6	219.4	33.0	28.71	1.25	25.83
3	36.5	43.4	224.7	32.2	30.91	1.25	26.92
4	36.6	42.5	227.2	26.8	34.21	1.35	30.15
5	36.9	41.8	228.8	27.5	34.87	1.25	30.16
10	36.4	41.5	228.8	26.3	34.85	1.25	30.12
15	36.2	41.5	228.7	26.6	37.91	1.20	31.53

TABLE II

Summary of Component Fatty Acids of Buffalo Colostrum (Expressed in Mole Per Cent)

Acids	Day of lactation			
	1st	2nd	3rd	10th
Butyric	7.4	7.4	8.9	12.1
Caproic	0.3	0.2	0.4	0.7
Caprylic	1.3	1.9	1.3	2.4
Capric	1.5	1.9	1.4	1.5
Lauric	1.7	2.8	2.3	1.8
Myristic	9.1	13.7	12.8	12.8
Palmitic	19.5	24.0	25.4	28.2
Stearic	15.9	13.8	12.4	11.5
Arachidic	0.9	0.6	0.8	0.7
Total	57.6	66.3	65.7	71.7
Decenoic	0.1	0.1	0.1	0.1
Dodecenoic	0.1	0.1	0.1	0.1
Tetradecenoic	0.4	0.6	0.6	0.6
Hexadecenoic	5.8	4.3	4.7	4.4
Oleic	34.1	27.0	27.8	21.6
Linoleic	0.7	0.2	0.1	0.2
C_{20-22} unsaturated	1.2	1.4	0.9	1.3
Total	42.4	33.7	34.3	28.3
Sum of acids up to C_{14}	21.9	28.7	27.9	32.1

marked progressive change in the chemical composition of the fat secreted by the animals. The butyrorefractometer reading and iodine value gradually decrease and the Reichert and saponification values of the fat increase. These observations were supplemented by the detailed analysis reported

in Table II, which indicates definite trends in the change in composition of the fat secreted by the buffalo as the period of lactation advances. There is a regular increase in the proportion of the fatty acids up to palmitic acid during the transition from the colostrum of the first days to the mature milk of the 10th day of lactation. The unsaturated fatty acids up to C_{14} are not appreciably affected during the transition period. There is a marked lowering in the amount of oleic and stearic acids of the fat from the 1st day after calving to the 10th day of lactation. However, the presence of large amounts of oleic acid in the colostral fat may be due to the fact that in the initial stages of lactation, when the general activity of the glands is less, the oxidation of the oleoglycerides is slow. As lactation advanced, the amount of oleic acid gradually decreases, this decrease being counterbalanced by an increase in the amount of saturated acids, mainly butyric, myristic, and palmitic. The results of the present investigation reveal an inverse relationship between the C_{18} acids on the one hand and the acids of lower molecular weight on the other, particularly of C_4, C_{14}, and C_{16} acids. Baldwin and Longenecker (2) have reported that there is no significant difference in the composition of the fat of colostrum and mature milk of cows, but we find that there is a gradual change in the composition of the colostrum fat of cows (3) as well as of buffaloes as lactation advances. The colostrum fat analyzed by them was from one cow and had been collected during the first 4 days after parturition, whereas we have analyzed the composite samples of fat collected from four buffaloes on each day during the progress of lactation.

SUMMARY

1. Samples of colostrum fat were collected from Murrah buffaloes during the progress of lactation and were studied for the chemical characteristics.

2. Fat from the composite mixtures of colostrum of the 1st, 2nd, and 3rd days and of mature milk of the 10th day of lactation was subjected to detailed ester fractionation for the study of fatty acid distribution.

3. The colostrum fat is found to differ in chemical composition from that of the normal butter fat of the buffalo.

4. The chief changes to be found were the gradual increase in the amount of butyric, myristic, and palmitic acids and a decrease in the amount of stearic and oleic acids, the decrease in the latter being more pronounced.

BIBLIOGRAPHY

1. Smith, J. A. B., and Dastur, N. N., *Biochem. J.*, **32**, 1868 (1938).
2. Baldwin, A. R., and Longenecker, H. E., *J. Biol. Chem.*, **155**, 407 (1944).
3. Anantakrishnan, C. P., Bhale Rao, V. R., and Paul, T. M., *Biochem. J.*, **40**, 293 (1946).

THE SYNTHESIS OF THE ISOMERS OF CYSTATHIONINE AND A STUDY OF THEIR AVAILABILITY IN SULFUR METABOLISM

By WILLIAM P. ANSLOW, Jr.,* SOFIA SIMMONDS, AND VINCENT
DU VIGNEAUD

*(From the Department of Biochemistry, Cornell University Medical College,
New York City)*

Considerable experimental evidence has accumulated indicating that cystathionine is an intermediate in the conversion of methionine to cystine (1–5). The following report describes the synthesis of the isomers of cystathionine and the testing of their ability to replace the sulfur-containing amino acids in the diet of the young rat.

The name cystathionine was suggested for the thio ether, S-(l-β-amino-β-carboxyethyl)-l-homocysteine (4). Since the present report is concerned with the four possible stereoisomers of the molecule, it is convenient to provide a stereochemical designation for the compounds. Since the isomer referred to above is related only to the natural amino acids and since it is dextrorotatory, it seems appropriate to designate it further as $l(+)$-cystathionine. According to this system S-(d-β-amino-β-carboxyethyl)-d-homocysteine, the optical isomer of $l(+)$-cystathionine, would be designated $d(-)$-cystathionine. The remaining two isomers are diastereoisomers of the first pair, and the name, *allocystathionine*, is suggested for them. Each of the allocystathionines is related to both the l- and d-amino acid series, but only one of the allocystathionines can yield l-cysteine on simple cleavage. Because biological interest in the compound has centered chiefly around its ability to serve as a precursor of cysteine, the cysteine-like moiety of the molecule is suggested as the stereochemical reference point. Thus, levorotatory S-(l-β-amino-β-carboxyethyl)-d-homocysteine, containing an l-cysteine moiety, may be designated $l(-)$-allocystathionine. Dextrorotatory S-(d-β-amino-β-carboxyethyl)-l-homocysteine, which similarly contains a d-cysteine moiety, may be designated $d(+)$-allocystathionine. The relationship between the four isomers is shown in the accompanying structures.

---

* Present address, Department of Physiology, New York University College of Medicine, New York.

```
      COOH                COOH                COOH                COOH
       |                   |                   |                   |
H₂N—C—H            H—C—NH₂            H—C—NH₂            H₂N—C—H
       |                   |                   |                   |
  H—C—H              H—C—H              H—C—H              H—C—H
       |                   |                   |                   |
  H—C—H              H—C—H              H—C—H              H—C—H
       |                   |                   |                   |
       S                   S                   S                   S
       |                   |                   |                   |
  H—C—H              H—C—H              H—C—H              H—C—H
       |                   |                   |                   |
  H—C—NH₂          H₂N—C—H            H—C—NH₂          H₂N—C—H
       |                   |                   |                   |
      COOH                COOH                COOH                COOH
 l(+)-Cysta-        d(−)-Cysta-      l(−)-Allocysta-    d(+)-Allocysta-
   thionine           thionine          thionine           thionine
```
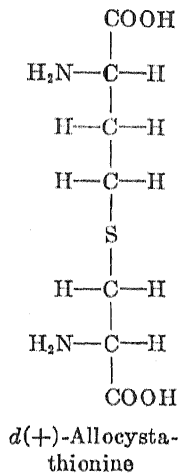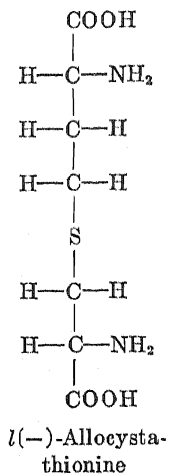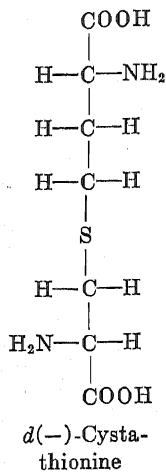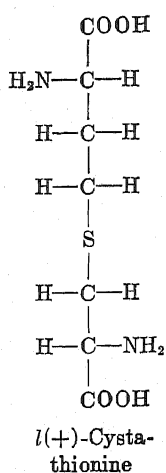
EXPERIMENTAL

S-Benzylhomocysteine was resolved and converted to the optically active isomers of homocystine (6). These homocystine isomers served as precursors of the homocysteines used in the formation of the cystathionine isomers. Serine was resolved by the method of Fischer and Jacobs (7) and converted to the optically active isomers of α-amino-β-chloropropionic acid, according to the directions of Fischer and Raske (8). Condensation of the respective isomers of homocysteine and α-amino-β-chloropropionic acid was carried out in the manner used to synthesize *l*(+)-cystathionine (2). Separation of the cystathionines from the accompanying homocystine was accomplished by recrystallization from a cyanide solution, according to the method originally devised by Brown and du Vigneaud for the separation of lanthionine from contamination with cystine (9).

d-Cystathionine—15.3 gm. of *d*-homocystine, $[\alpha]_D^{22} = -79.6°$, were reduced in liquid ammonia with metallic sodium, according to the directions of du Vigneaud and Patterson (6). On completion of the reduction, the flask was removed from the cooling bath and the ammonia was allowed to evaporate. When ammonia no longer issued, the flask was evacuated for ½ hour at water pump pressure and filled with nitrogen. 50 cc. of oxygen-free water were added, and the flask was evacuated again to remove excess ammonia and refilled with nitrogen. As in the earlier synthesis of the mixed isomers of cystathionine (10), the homocysteine was used without isolation.

17.9 gm. of methyl *d*-α-amino-β-chloropropionate hydrochloride were hydrolyzed by heating to 100° with 180 cc. of 20 per cent HCl. The clear solution which resulted was evaporated under reduced pressure,

redissolved in water, and evaporated again. A thick mush of crystals resulted, and this was dissolved in 25 cc. of oxygen-free water.

The flask containing the homocysteine was equipped with a stirrer and immersed in a water bath kept at approximately 60°. The solution of α-amino-β-chloropropionic acid hydrochloride was added portionwise during a period of 2 hours. When the reaction mixture became acid to phenolphthalein paper, a few pellets of solid KOH were added. After the last addition of α-amino-β-chloropropionic acid, the flask was allowed to remain in the water bath from 9 to 10 hours. At the end of this time, 200 cc. of oxygen-free water were added to dissolve the precipitated salt, and the pH of the solution was adjusted to pH 6.0 with concentrated HCl. The flask was filled with nitrogen and allowed to stand in the refrigerator overnight.

The resulting precipitate was separated and suspended in 300 cc. of water. 25 cc. of concentrated HCl were added. The resultant dark colored solution was clarified by filtration through a layer of Darco and neutralized to pH 6.0 with concentrated NH$_4$OH. The crystalline precipitate was filtered and washed with water. The dried product weighed 11.2 gm. A sample showed a strong sodium cyanide-nitroprusside test for disulfide.

To separate the $d(-)$-cystathionine from homocystine, the above product was suspended in 200 cc. of water and dissolved by the addition of concentrated NH$_4$OH. The solution was filtered, and to the filtrate 0.5 gm. of NaCN was added. After 30 minutes the solution was neutralized to pH 6.0 with glacial acetic acid; crystallization occurred spontaneously. The crystals were collected by filtration and the cyanide treatment was repeated. After the final recrystallization, 9.2 gm. of product were obtained. A sample of this product showed a faint cyanide-nitroprusside test. Analysis of a portion of it for homocystine by the Kassell and Brand modification (11) of the Folin-Marenzi method (12) showed the presence of less than 1 per cent of homocystine. A 1 per cent solution of the compound in 1 N HCl possessed a rotation of $[\alpha]_D^{21.5} = -23.5°$, which compares favorably with the value of $[\alpha]_D^{20} = +23.7°$ for $l(+)$-cystathionine (2).

C$_7$H$_{14}$O$_4$N$_2$S.   Calculated.   N 12.60, S 14.42
222.3   Found.   " 12.80, " 14.89

Dibenzoyl-d-cystathionine—200 mg. of $d(-)$-cystathionine were suspended in 10 cc. of water in a 50 cc. ground glass-stoppered flask and dissolved by the addition of NaOH. The flask was shaken in an ice bath and 0.35 cc. of benzoyl chloride was added in three portions. Each addition was followed by vigorous shaking until the odor of benzoyl chloride disap-

peared. The reaction mixture was kept alkaline to phenolphthalein by the addition of more alkali. After the last addition of benzoyl chloride, the reaction mixture was allowed to stand at room temperature for 1 hour and then was made acid to Congo red paper by the addition of concentrated HCl. An oily precipitate separated which soon solidified. After the flask had been cooled in the refrigerator overnight, the solid mass was separated by filtration and washed with water.

The product was dissolved in 50 cc. of 70 per cent alcohol, heated, and the hot solution was filtered through a layer of Darco. The clear filtrate was evaporated *in vacuo* until crystals appeared. After the flask was cooled overnight, the crystalline precipitate was filtered, washed with 5 cc. of cold 70 per cent alcohol, and dried at 80°. This dibenzoyl-*d*-cystathionine melted[1] at 232–234°. The melting point recorded for dibenzoyl-*l*-cystathionine is 229° (2).

$C_{21}H_{22}O_6N_2S$. Calculated. N 6.51, S 7.45
430.5   Found.   " 6.74, " 7.27

l(−)-*Allocystathionine*—This isomer was synthesized from 15.3 gm. of *d*-homocystine, $[\alpha]_D^{22} = -79.6°$, and 17.9 gm. of methyl *l*-α-amino-β-chloropropionate hydrochloride. The product obtained from this coupling reaction was suspended in 300 cc. of water and dissolved by the addition of concentrated HCl. After clarification by filtering through a layer of Darco, the solution was neutralized to pH 6.0. After 24 hours in the cold room, the crystalline precipitate was collected. The product was washed with water and dried at 80°. A yield of 12.6 gm. was obtained. This product showed a strong cyanide-nitroprusside test.

The product was recrystallized three times by precipitation from an alkaline cyanide solution, as described above for *d*(−)-cystathionine. This treatment yielded 3 gm. of product which still showed a positive cyanide-nitroprusside test, but analysis for homocystine showed the presence of less than 1 per cent of homocystine. A 1 per cent solution of the *l*(−)-allocystathionine in 1 N HCl possessed a rotation of $[\alpha]_D^{21} = -25.0°$.

$C_7H_{14}O_4N_2S$. Calculated. N 12.60, S 14.42
222.3   Found.   " 12.03, " 14.68

Dibenzoyl-l-allocystathionine—This derivative was prepared from 200 mg. of *l*(−)-allocystathionine by the procedure described for dibenzoyl-*d*-cystathionine. The recrystallized product melted at 186–187°.

$C_{21}H_{22}O_6N_2S$. Calculated. N 6.51, S 7.45
430.5   Found.   " 6.55, " 7.68

[1] All melting points are corrected capillary melting points.

$d(+)$-*Allocystathionine*—This isomer was synthesized from 15.3 gm. of l-homocystine, $[\alpha]_D^{22} = +74.0°$, and 17.9 gm. of methyl d-α-amino-β-chloropropionate hydrochloride. The product was treated as described previously and, after three precipitations from alkaline cyanide, 4 gm. of product were obtained which were shown to contain less than 1 per cent of homocystine by analysis. A 1 per cent solution of the $d(+)$-allocysta-thionine in 1 N HCl possessed a rotation of $[\alpha]_D^{21} = +24.5°$.

$C_7H_{14}O_4N_2S$.   Calculated.   N 12.60, S 14.42
222.3   Found.   " 12.28, " 14.39

Dibenzoyl-d-allocystathionine—This derivative was prepared from 200 mg. of $d(+)$-allocystathionine according to the procedure described for dibenzoyl-d-cystathionine. After two recrystallizations from 70 per cent alcohol, the product melted at 189°.

$C_{21}H_{22}O_6N_2S$.   Calculated.   N 6.51, S 7.45
430.5   Found.   " 6.46, " 7.57

Feeding Experiments

For the study of the availability of the isomers of cystathionine for the replacement of cystine in the diet of the growing white rat, the diet was the same as that used with $l(+)$-cystathionine (2). Supplementation of this diet with either cystine or $l(+)$-cystathionine had been shown to permit growth (2). The protein nitrogen of the diet was supplied by a mixture of pure amino acids patterned after the amino acid mixture of Rose and Rice (13). The diet had the following composition: amino acid mixture (exclusive of the sulfur-containing amino acids) 21.3, sucrose 44.7, Crisco 30.0 (including fat-soluble vitamin supplement), and salt mixture (Osborne and Mendel (14)) 4.0 parts, respectively.

The sulfur-containing amino acids were added to the amino acid mixture and an equal weight of sucrose was omitted from the diet. The fat-soluble vitamins were furnished in a corn oil solution, 4 cc. of which were added to the fat component of 1 kilo of diet. The source and quantities of these vitamins have been described previously (15). 0.5 cc. of a solution containing the water-soluble vitamins was fed twice daily. 1 cc. of this solution contained 20 γ of thiamine chloride, 20 γ of riboflavin, 20 γ of nicotinic acid, 20 γ of pyridoxine hydrochloride, 200 γ of calcium dl-pantothenate, 5 mg. of inositol, and 25 mg. of choline chloride. The diets and water were allowed *ad libitum*, and the animals were weighed every 4 days.

For the basal diet in the experiments recorded in Table I and Fig. 1 methionine was added to form 0.2 per cent of the diet. This amount of methionine was such that growth was not permitted on the basal diet, but

when sufficient cystine was added growth would result.   The cystathionine isomers and cystine were added to the basal diet in amounts indicated in Table I.   The growth curves for the animals used in these experiments are shown in Fig. 1.   The daily food consumption is given in Table I.

TABLE I

Food Consumption of Rats Fed Cystathionines in Place of Cystine

Rat No. and sex	Supplement to basal diet	Experimental period	Average daily food consumption
		days	*gm.*
1435 ♀	None	10	2.7
	0.74% *l*(−)-allocystathionine	20	5.5
	None	7	4.3
1436 ♀	"	10	3.1
	0.74% *l*(−)-allocystathionine	20	5.8
	0.74% *d*(+)-allocystathionine	7	4.0
1440 ♀	None	10	2.9
	0.74% *d*(+)-allocystathionine	20	2.9
	None	2	*
1444 ♂	"	10	3.3
	0.74% *d*(+)-allocystathionine	20	5.1
	None	7	4.9
1437 ♂	"	10	4.2
	0.74% *d*(−)-cystathionine	20	4.7
	None	7	4.9
1442 ♂	"	10	3.4
	0.74% *d*(−)-cystathionine	20	4.2
	None	7	5.4
1439 ♂	"	10	3.8
	0.74% *l*(+)-cystathionine	20	6.1
	0.74% *d*(−)-cystathionine	7	5.0
1441 ♀	None	10	2.8
	0.4% *l*-cystine	20	4.0
	None	7	5.3
1443 ♀	"	17	3.7
	0.4% *l*-cystine	13	5.6
	None	7	6.1
1438 ♂	"	37	4.7

* The animal died 2 days after being placed on the basal diet.   The food consumption was negligible.

The curves in Fig. 1 show that the addition of 0.4 per cent of cystine to the basal diet made it adequate for growth.   In agreement with the results of earlier experiments, substitution of an equivalent quantity (0.74 per cent) of *l*(+)-cystathionine permitted growth approximately equal to that obtained with a cystine supplement.   Of the other stereoisomers of

cystathionine, only one supported growth in place of cystine. A supplement of 0.74 per cent of $l(-)$-allocystathionine gave growth at a rate
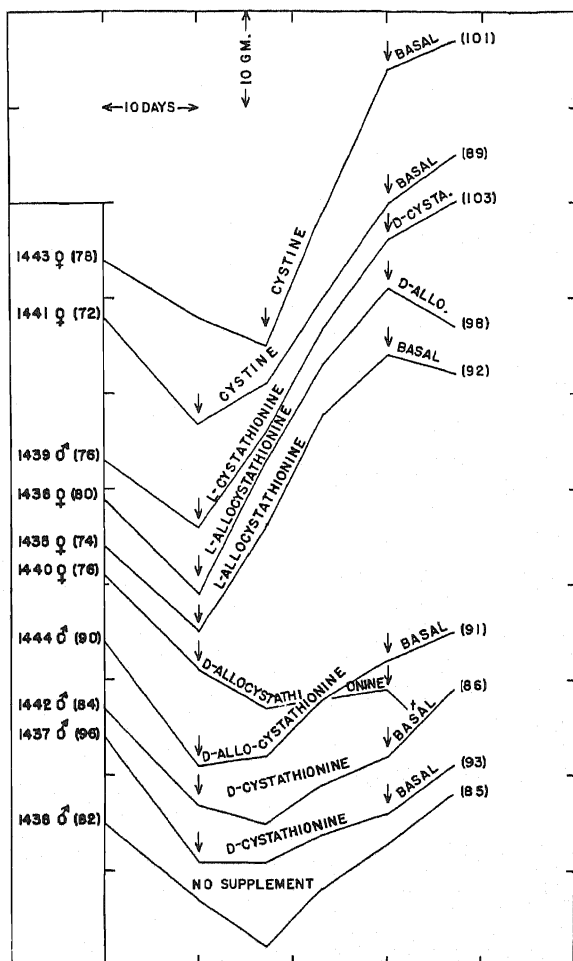
FIG. 1. Growth curves for rats fed cystathionines in place of cystine. The number and sex of the animals (litter mates) are indicated at the extreme left; the initial and final weights are in parentheses; a dagger indicates that the animal died. The dietary supplement is noted along the curves; the arrows indicate the times at which the diet was changed. The basal diet contains 0.2 per cent methionine.

comparable to that of an equivalent amount of cystine or $l(+)$-cystathionine. The parallel growth curves of Rats 1435, 1436, and 1441 suggest that $l(-)$-allocystathionine can support growth as well as an equivalent quantity of cystine. Supplementation of the basal diet with either

$d(-)$-cystathionine or $d(+)$-allocystathionine resulted in no better growth than that of the control animal.

TABLE II

Food Consumption of Rats Fed $l(-)$-Allocystathionine in Place of Methionine

Rat No. and sex*	Supplement to basal diet†	Experimental period	Average daily food consumption	Remarks on feeding‡
		days	*gm.*	
1508 ♂	Choline	5	3.0	
	0.46% *dl*-homocystine + choline	17	6.7	
	Choline	11	4.2	
2607 ♀	"	4	3.3	
	0.46% *dl*-homocystine + choline	15	4.9	Restricted
1510 ♀	Choline	5	2.6	
	0.74% *l*(−)-allocystathionine + choline	18	4.9	
	0.46% *dl*-homocystine + choline	11	6.8	
2608 ♀	Choline	4	2.3	
	0.74% *l*(−)-allocystathionine + choline	15	4.2	
2609 ♂	Choline	4	2.8	
	0.74% *l*(−)-allocystathionine + choline	15	4.5	
1511 ♀	None	5	3.2	
	0.74% *l*(−)-allocystathionine	14	3.4	
2611 ♀	Choline	4	3.0	
	0.74% *l*(−)-allocystathionine	10	4.4	
	0.74% "	5	0.8	
2610 ♂	Choline	4	2.5	
	0.74% *l*(−)-allocystathionine	7	4.6	
	0.74% "	4	0.8	
2606 ♀	Choline	4	2.8	
	0.46% *dl*-homocystine	10	4.6	Restricted
	0.46% "	5	2.1	
2605 ♀	Choline	19	3.0	

* Rats 1508 to 1511 inclusive were purchased from the Rockland Farms; Rats 2605 to 2611 inclusive were litter mates from our colony.

† Data in this column note the presence or absence of 25 mg. of choline chloride (per day) in the vitamin supplement.

‡ Unless otherwise noted, feeding was *ad libitum*.

Cleavage of $l(-)$-allocystathionine to l-cysteine would offer a simple explanation of its ability to support growth in place of cystine. However, subsequent studies *in vitro* showed that the cleavage of $l(-)$-allocystathionine by liver tissue presumably yielded d-homocysteine.[2] Since

[2] Anslow, W. P., Jr., and du Vigneaud, V., unpublished data.
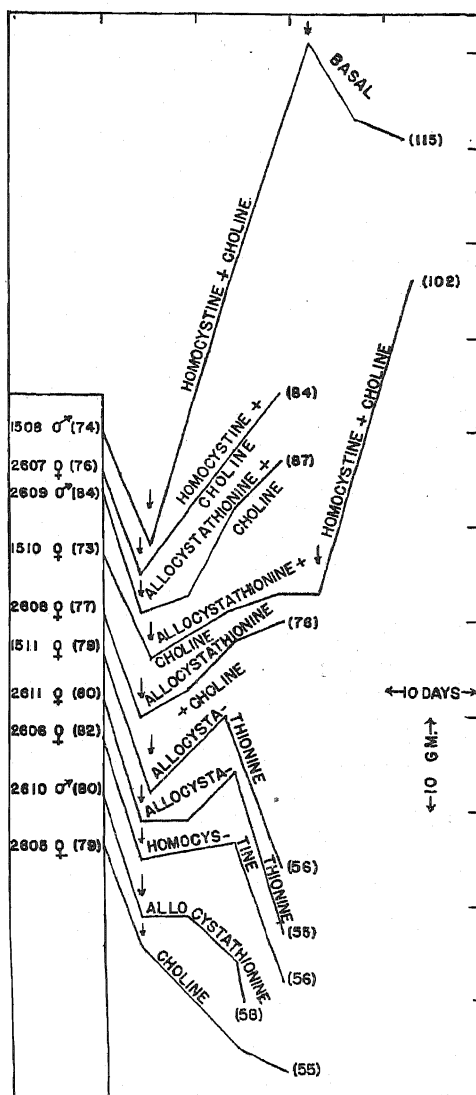
FIG. 2. Growth curves for rats fed $l(-)$-allocystathionine in place of methionine. The number and sex of the animals are indicated at the extreme left; the initial and final weights are in parentheses. The dietary supplement is noted along the curves; the arrows indicate the times at which the diet was changed. The basal diet contains 0.4 per cent cystine.

d-homocystine can support growth in place of cystine (16), cleavage to homocysteine is the more probable explanation of the ability of $l(-)$-allocystathionine to support growth. If $l(-)$-allocystathionine is cleaved

to d-homocysteine in the intact animal, then $l(-)$-allocystathionine supplemented with adequate dietary choline might be expected to support growth in place of methionine.

To test the latter hypothesis, a series of rats was placed on a basal diet containing 0.4 per cent cystine but no methionine, a diet used to test the same hypothesis in the case of $l(+)$-cystathionine (2), under which conditions $l(+)$-cystathionine did not support growth. The diet had the same composition as the one used above except with respect to the sulfur-containing amino acids. dl-Homocystine or $l(-)$-allocystathionine was added to this diet in the amounts indicated in Table II. Growth curves for this series of animals are shown in Fig. 2.

Supplementation of the basal diet with dl-homocystine made the diet adequate for growth. Supplementation with an equivalent quantity of $l(-)$-allocystathionine likewise brought about growth, but not to so great an extent as did the dl-homocystine supplement. Data in Table II show that the food consumption of animals fed the $l(-)$-allocystathionine ranges from 4.4 to 4.9 gm. per day, whereas the food consumption of animals fed homocystine was approximately 6.7 gm. per day. When the intake of Rat 2607 (receiving the homocystine supplement) was restricted to 4.9 gm. per day, the rate of growth of this animal was comparable to that of the animal fed $l(-)$-allocystathionine.

When the choline was removed from the vitamin supplement, Rats 1511, 2611, and 2606 grew for a short time. However, all the rats began to lose weight precipitously from the 7th to the 10th day of the choline-free diet. Palpation indicated enlarged kidneys in Rat 2610 on the 12th day, in Rat 2611 on the 16th day, and in Rat 2606 on the 18th day. At autopsy gross examination of these three animals showed large mottled kidneys in Rat 2606, and marked enlargement and hyperemia in Rats 2610 and 2611. Enlarged kidneys were not felt in Rat 1511 and growth was resumed when choline was again included in the vitamin supplement.

SUMMARY

The three previously uncharacterized isomers of cystathionine have been synthesized and characterized. A stereochemical nomenclature for these compounds is suggested.

On a cystine-free, methionine-restricted diet, $l(-)$-allocystathionine is able to support growth in the young rat, behaving in this respect like $l(+)$-cystathionine. However, on a diet adequate with respect to cystine but deficient in methionine, $l(-)$-allocystathionine supports growth in the young rat when fed together with choline, in contrast to the behavior of $l(+)$-cystathionine under these conditions.

$d(-)$-Cystathionine and $d(+)$-allocystathionine do not support growth on a cystine-free, methionine-restricted diet.

The authors wish to acknowledge their appreciation to Dr. Julian R. Rachele and Mr. Roscoe C. Funk, Jr., for the microanalyses reported in this paper.

BIBLIOGRAPHY

1. Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).
2. du Vigneaud, V., Brown, G. B., and Chandler, J. P., *J. Biol. Chem.*, **143**, 59 (1942).
3. Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 559 (1942).
4. Binkley, F., and du Vigneaud, V., *J. Biol. Chem.*, **144**, 507 (1942).
5. Binkley, F., *J. Biol. Chem.*, **155**, 39 (1944).
6. du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935).
7. Fischer, E., and Jacobs, W. A., *Ber. chem. Ges.*, **39**, 2942 (1906).
8. Fischer, E., and Raske, K., *Ber. chem. Ges.*, **40**, 3717 (1907).
9. Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **140**, 767 (1941).
10. Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **137**, 611 (1941).
11. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938).
12. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
13. Rose, W. C., and Rice, E. E., *J. Biol. Chem.*, **130**, 305 (1939).
14. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
15. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135**, 223 (1940).
16. Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **109**, 477 (1935).

A FURTHER INVESTIGATION OF THE ABILITY OF SARCO-SINE TO SERVE AS A LABILE METHYL DONOR*

By VINCENT du VIGNEAUD, SOFIA SIMMONDS, and MILDRED COHN

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

It is the purpose of the present paper to report a study of transmethylation with isotopic sarcosine. The sarcosine used in these experiments was labeled with deuterium in the methyl group and also with N^{15}. The compound was fed to four adult rats for 7 days as a supplement to an otherwise methyl-free diet containing homocystine. At the end of this time, the rats were sacrificed and choline, creatine, glycine, and glutamic acid were isolated from the tissues of each rat. In order to determine the relative efficacy of sarcosine as a methyl donor, a parallel experiment under identical conditions was carried out with isotopic choline. In the latter compound, only the methyl groups were labeled. The data from these experiments are summarized in Table I.

As shown in Table I, sarcosine was not an effective methyl donor for the synthesis of choline and creatine, although a small fraction of the methyl groups of these tissue constituents was derived from the dietary sarcosine. About 1 to 2 per cent of the methyl groups of choline and about 0.5 per cent of the methyl group of creatine were derived from the sarcosine in 7 days. When an amount of methyl groups equivalent to that supplied as isotopic sarcosine was fed in the form of deuteriocholine, approximately 22 per cent of the tissue choline-methyl and approximately 2 per cent of the creatine-methyl groups were derived from the diet.

In order to eliminate variations due to differences in body weight of the rats and differences in amounts of test compound ingested, the data may be recalculated on the basis of mM of test compound ingested per 100 gm. of body weight. When the data are recalculated in this manner (Table II), the creatine N^{15} values resulting from the feeding of isotopic sarcosine appear to exhibit a random fluctuation within ± 10 per cent. On the other hand, the variation of deuteriomethyl concentration in the creatine is of a different order of magnitude; approximately 70 per cent more methyl groups of creatine have been derived from dietary sarcosine in the female rats than in the male rats. A similar recalculation of the data from the feeding of choline (Table II) shows no significant difference between the

47

TABLE I

Feeding Experiments with Isotopic Sarcosine Hydrochloride (82.9 Atom Per Cent D in Methyl Group, 4.17 Atom Per Cent Excess N^{15}) and with Deuteriocholine Chloride (83.1 Atom Per Cent D in Methyl Groups) for 7 Days

Rat No. and sex	Compound fed	Change in body weight	Choline		Creatine			Glycine	Glutamic acid
			Total methyl	Nitrogen	Methyl	Total nitrogen	Sarcosine nitrogen*	Nitrogen	Nitrogen
		gm.							
1752 ♂	N^{15} deuteriosarco-	364–330	1.11 ± 0.08	1.39 ± 0.07	0.29 ± 0.10	1.03 ± 0.07	2.5	1.94 ± 0.07	0.50 ± 0.07
1753 ♂	sine hydrochloride	350–310	1.39 ± 0.08	1.51 ± 0.07	0.43 ± 0.10	1.03 ± 0.07	2.5	2.44 ± 0.07	0.48 ± 0.07
1755 ♀		328–293	1.46 ± 0.04	1.53 ± 0.07	0.63 ± 0.10	1.25 ± 0.07	3.0	2.66 ± 0.07	0.53 ± 0.07
1757 ♀		278–259	2.16 ± 0.08	1.92 ± 0.07	0.82 ± 0.10	1.41 ± 0.07	3.4	2.88 ± 0.07	0.65 ± 0.07
1754 ♂	Deuteriocholine	355–344	21.3 ± 0.18		2.07 ± 0.24				
1760 ♀	chloride	296–288	22.6 ± 0.18		2.12 ± 0.24				

Per cent of methyl or N in isolated compounds derived from test compounds

* N^{15} of sarcosine moiety of creatine calculated on basis of N^{15} distribution data of Bloch and Schoenheimer (1).

male and the female rat.   The sarcosine results with respect to sex difference will bear further experimental investigation.

A comparison of the percentage of methyl groups[1] and nitrogen in the isolated choline derived from the fed sarcosine shows that the sarcosine molecule is not converted to choline as a whole.   Of even greater interest is a similar comparison in the isolated creatine which reveals that the sarcosine molecule is not converted intact to creatine but first is demethylated to yield glycine.   This is in agreement with the experimental findings of Bloch and Schoenheimer (1, 2) and of Borsook and Dubnoff (3).

TABLE II

Data from Table I Recalculated on Basis of mM of Test Compound Ingested Per 100 Gm. of Body Weight

Rat No. and sex	mM test compound ingested in 7 days per 100 gm. body weight	Per cent of methyl or N in isolated compounds derived from test compounds					
		Choline		Sarcosine moiety of creatine		Glycine	Glutamic acid
		Total methyl	Nitrogen	Methyl	Nitrogen	Nitrogen	Nitrogen
N[15] deuteriosarcosine hydrochloride							
1752 ♂	2.29	0.485	0.606	0.127	1.08	0.846	0.218
1753 ♂	2.56	0.543	0.590	0.168	0.97	0.953	0.188
1755 ♀	2.56	0.570	0.597	0.246	1.17	1.04	0.207
1757 ♀	3.14	0.688	0.611	0.261	1.08	0.918	0.207
Deuteriocholine chloride							
1754 ♂	2.45*	8.70		0.845			
1756 ♀	2.93*	7.71		0.724			

* This figure represents the number of milliequivalents of methyl groups rather than of choline.

With the data available from the present study with sarcosine, it is now possible to compare the three N-methylglycine derivatives, sarcosine, dimethylglycine, and betaine, as methyl donors.   Although a direct comparison among these compounds cannot be made because the sarcosine was fed to adult rats, whereas the dimethylglycine and betaine were fed to growing rats (4), the relative activity of each compound can be evaluated nevertheless by comparison with choline fed under the same conditions.   Such a

[1] The figures in Tables I and II represent the percentage of three methyl groups of choline derived from sarcosine. If one considered only one methyl group of choline which would be necessary for a direct conversion of sarcosine to choline, the value would be three times as high.

comparison shows that betaine (4) is by far the most active of the three glycine derivatives. Dimethylglycine (4) and sarcosine are both relatively inactive. This is in agreement with the fact that betaine is the only one which can replace choline in the growth tests (5, 6).

<div align="center">EXPERIMENTAL</div>

Synthesis of N^{15} Deuteriosarcosine Hydrochloride $(CD_3N^{15}HCH_2COOH \cdot HCl)$—Isotopic sarcosine hydrochloride was prepared from N^{15} glycine (4) and deuteriomethyl iodide (7) by the method of Fischer and Bergmann (8).

Analysis—

<div align="center">

N^{15} deuteriosarcosine hydrochloride. Calculated.[2] N 10.92

Found. " 10.53

</div>

The compound contained 82.9 atom per cent excess deuterium in the methyl group and 4.17 atom per cent excess N^{15}.

Feeding of N^{15} Deuteriosarcosine—Four adult rats, two males and two females, ranging in weight from 280 to 360 gm., were used. The isotopic sarcosine hydrochloride, with sufficient $NaHCO_3$ to neutralize it, was incorporated in a diet containing 1.25 per cent homocystine (4) and was the only possible source of labile methyl groups in the diet. For the first 4 days of the feeding period, 2.2 gm. of isotopic sarcosine hydrochloride were added per 100 gm. of diet. Since the daily food intake was somewhat lower than expected, it was necessary to give additional isotopic sarcosine hydrochloride, neutralized with $NaHCO_3$, in the aqueous solution of B vitamins (4) in order to maintain the total intake of sarcosine hydrochloride at approximately 150 mg. per rat daily.

To avoid giving the sarcosine in both the diet and the B vitamin supplement, during the last 3 days of the experiment 3.2 gm. of the isotopic sarcosine hydrochloride were added per 100 gm. of diet. During the entire 7 day feeding period, each rat ingested a total of approximately 1.03 gm. of test compound, which corresponds to a daily intake of sarcosine equivalent in methyl groups to 58 mg. of choline chloride.

At the end of the 7 day experimental feeding period, choline was isolated as the chloroplatinate and creatinine as creatinine potassium picrate from the tissues of the sacrificed animals (7); for the N^{15} analyses, picric acid was removed from creatinine potassium picrate (4). Glycine and glutamic acid also were isolated from the tissue proteins. Glycine was isolated as the trioxalatochromiate, which was converted, for the purpose of analyses, to carbobenzoxyglycine (4, 9). Glutamic acid was isolated as glutamic acid hydrochloride (4, 10). The pertinent data from this experiment are summarized in Table I.

---

[2] All calculated values are based on increased molecular weights due to deuterium in the molecule.

Analyses—Choline chloroplatinate

      Rat 1752. Calculated,[2] Pt 31.7; found, Pt 31.4

      " 1753.   "   " 31.7; "   " 31.5

      " 1755.   "   " 31.6; "   " 31.7

      " 1757.   "   " 31.6; "   " 31.2

Carbobenzoxyglycine

      Rat 1752. Calculated, N 6.70; found, N 6.41

      " 1753.   "   " 6.70; "   " 6.58

      " 1755.   "   " 6.70; "   " 6.62

      " 1757.   "   " 6.70; "   " 6.58

Glutamic acid hydrochloride

      Rat 1752. Calculated, N 7.63; found, N 7.41

      " 1753.   "   " 7.63; "   " 7.64

      " 1755.   "   " 7.63; "   " 7.46

      " 1757.   "   " 7.63; "   " 7.67

Feeding of Deuteriocholine Chloride $((CD_3)_3N(Cl)CH_2CH_2OH)$—Two adult rats, one male and one female weighing 355 and 296 gm. respectively, were maintained on the same homocystine diet (4) as used in the preceding experiment. 29 mg. of deuteriocholine chloride (7) in the aqueous solution of B vitamins (4) were fed twice daily to each rat. The deuteriocholine chloride (7) contained 83.1 atom per cent excess deuterium in the methyl groups. After 7 days, the rats were sacrificed and tissue choline and creatine were isolated (7). The data from this experiment are summarized in Table I.

Analyses—Choline chloroplatinate

      Rat 1754. Calculated,[2] Pt 31.5; found, Pt 31.2

      " 1756.   "   " 31.5; "   " 31.7

SUMMARY

The ability of sarcosine to serve as a methyl donor for creatine and choline has been investigated by feeding adult rats N^{15} deuteriosarcosine $(CD_3N^{15}HCH_2COOH)$. Transmethylation from dietary sarcosine to tissue choline and creatine was observed, but the apparent rate of these reactions was slow, as compared to the rate at which methyl groups of dietary deuteriocholine appear in the choline and creatine of the tissues.

BIBLIOGRAPHY

1. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **138**, 167 (1941).
2. Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 99 (1940).
3. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138**, 389 (1941).

4. du Vigneaud, V., Simmonds, S., Chandler, J. P., and Cohn, M., *J. Biol. Chem.*, **165**, 639 (1946).
5. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
6. Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 373 (1942).
7. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).
8. Fischer, E., and Bergmann, M., *Ann. Chem.*, **398**, 96 (1913).
9. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **122**, 577 (1937–38).
10. Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1940).

THE DISTRIBUTION OF NIACINAMIDE AND NIACIN IN NATURAL MATERIALS*

By W. A. KREHL, J. DE LA HUERGA, C. A. ELVEHJEM, AND E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

Recognition of the importance of niacin in the prevention and cure of pellagra and the development of microbiological assays for this vitamin (1, 2) stimulated extensive studies on its distribution in foods and feeds (3–5).

There is, however, very little information concerning the distribution of nicotinamide in natural materials, although it is stated that "free nicotinic acid apparently does not occur in the living organism but is found in the urine of animals" (6). The significance of nicotinamide in natural materials is further indicated by the fact that it is contained in coenzymes I and II. Although niacin and niacinamide show no difference in antipellagra activity, high levels of the two compounds do show different physiological effects; namely, the flushing effect of niacin.

The development of a differential assay for niacin and niacinamide (7) has made it possible to study the distribution of these two compounds in a variety of natural materials, and a report of this study follows.

EXPERIMENTAL

Although it has been clearly shown that the test organism *Leuconostoc mesenteroides* (American Type Culture Collection No. 9135) responds to nicotinic acid but not to nicotinamide at low concentrations, it was considered desirable to reexamine the conditions under which nicotinamide is hydrolyzed to nicotinic acid and thus made available to the organism.

The method of assay as reported by Johnson (7) was followed in detail, except that the basal medium was modified as follows: glucose was reduced from 0.3 to 0.2 gm. per tube, pyridoxal was increased from 0.5 to 1.0 γ per tube, and uracil was added at a level of 0.1 mg. per tube. Very good standard curves were obtained with this method and a representative one is shown in Fig. 1.

To determine the best method for sample preparation and the hydrolysis

of nicotinamide to nicotinic acid, various reagents were used at an autoclaving temperature of 121° for a period of 1 hour.   The results of these experi-
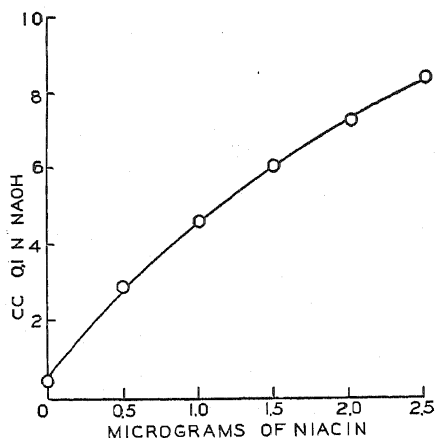
FIG. 1. Standard curve with *Leuconostoc mesenteroides*

TABLE I

Hydrolysis of Niacinamide

Method of hydrolysis*	Recovery of†	
	50 γ niacin	50 γ niacinamide
	per cent	*per cent*
H₂O..........................	100	0
0.6 N H₂SO₄ (Ba(OH)₂-neutralized)........................		71
1.0 N H₂SO₄ (Ba(OH)₂-neutralized)........................		77
1.0 N H₂SO₄ (NaOH-neutralized)		83
0.6 " HCl (NaOH-neutralized)..	100	95–100
1.0 " " " " ..	100	100
0.1 " NaOH (HCl ")..	100	100
0.25 N NaOH (HCl-neutralized).	100	100
0.5 " " " " .	100	100

* All hydrolysis experiments were conducted by autoclaving at 121° for 1 hour.

† Recovery studies made with *Leuconostoc mesenteroides* (American Type Culture Collection No. 9135).

ments are compiled in Table I and show that H₂SO₄ hydrolysis, followed by removal of the sulfate ion with Ba(OH)₂, or its neutralization with NaOH, resulted in low recoveries of nicotinamide.   (The nicotinamide concentrations used for the recovery experiments were checked against the standard

niacin assay with *Lactobacillus arabinosus* (2).)   The use of HCl or NaOH, however, proved very satisfactory for the complete hydrolysis of niacinamide and recovery of the niacin formed.   These results are somewhat in variance with the previously reported work (7) in which it was stated that HCl could not be used for the hydrolysis, since the NaCl formed on neutralization with NaOH inhibited the growth of the bacteria.   We have found no growth inhibition from concentrations of NaCl up to and including 2 per cent.   This level of NaCl exceeds the concentration which would be found in any of the 1 N HCl-treated samples reported in this study.   The reason for this difference might reside in the thoroughness with which the HCl was removed from the acid-hydrolyzed casein used in the basal medium.   Inasmuch as excellent recoveries of niacin were obtained with 1 N HCl hydrolysis of nicotinamide and since this reagent is much more convenient to use than H_2SO_4, it has been employed for the studies which follow.

The samples chosen for analysis (Table II) were selected largely on the basis of their importance as human and animal foods.   The analysis of rat tissues was chosen since the tissues represent readily available and standard animal tissues.

A standard method, based on the above studies, was adopted for preparing the samples for analysis.   This consisted of autoclaving 1 gm. of sample for 1 hour at 121° with 50 volumes of either H_2O or 1 N HCl.   In order to check the validity of the *Leuconostoc mesenteroides* assay, and particularly to check the effectiveness of H_2O extraction of niacin under these conditions, all samples were prepared as above and assayed for niacin with *Lactobacillus arabinosus*, which does not distinguish between niacin and niacinamide. The nicotinamide content of the sample was found by using the difference between the assay value obtained with *Leuconostoc mesenteroides* before acid hydrolysis with 1 N HCl (*i.e.*, H_2O-treated samples) and the value obtained after acid hydrolysis.   The analytical values obtained with both *Lactobacillus arabinosus* and *Leuconostoc mesenteroides*, together with the per cent of apparent nicotinamide, are given in Table II.

The validity of the *Leuconostoc mesenteroides* assay for total niacin activity is clearly indicated by the remarkable agreement with the values obtained with *Lactobacillus arabinosus* with 1 N HCl for hydrolysis.   The efficacy of H_2O extraction of the various materials is evident in all samples except perhaps for wheat bran, wheat middlings, and white potatoes.   The differences in these cases are probably not due to inadequate extraction but rather to incomplete hydrolysis of a precursor of nicotinic acid which has been demonstrated in these products (8).   Incidentally, it is also clear that this precursor, in addition to nicotinamide, is unavailable to *Leuconostoc mesenteroides*, since the water-treated samples indicated above show a lower value with this organism than with *Lactobacillus arabinosus*.   Hy-

drolysis of these particular samples with 1 N HCl allows full and comparable activity for both organisms.

TABLE II

Distribution of Niacin and Niacinamide in Natural Materials

Material analyzed*	Lactobacillus arabinosus Total niacin		Leuconostoc mesenteroides		Niacinamide (total† minus free)	Per cent niacinamide of total niacin
	H_2O extract	1 N HCl extract	Total niacin, 1 N HCl extract	Free niacin, H_2O extract		
	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	
Hard wheat.............	45.5	46.5	49.7	34.7	15.0	30.0
Soft "	42.8	48.5	52.0	37.2	14.8	28.5
Wheat bran.............	202	246	245	197	48.0	19.6
" middlings........	138	172	179	111	68.0	38.0
" germ	41.5	41.5	43.5	22.8	20.7	47.5
White corn............	22.7	25.7	25.5	20.2	5.3	20.8
Yellow "	22.2	22.8	27.1	25.0	2.1	7.8
Corn bran.............	55.0	55.8	57.0	27.5	29.5	51.8
" gluten...........	71.5	79.0	89.3	70.7	18.6	20.8
Barley.................	59.2	65.7	68.3	43.0	25.3	37.0
Oats..................	6.4	6.8	7.4	6.5	0.9	12.2
Rye...................	11.2	10.8	10.7	8.5	2.2	20.5
Polished rice..........	15.0	18.2	20.9	17.2	3.7	17.7
White potatoes........	27.8	54.1	51.1	14.9	36.2	70.8
Soy bean oil meal......	19.9	20.6	25.3	12.6	12.7	50.1
Linseed " "	44.5	51.6	59.1	29.0	30.1	51.0
Cow peas..............	19.1	21.6	26.5	15.9	10.6	40.0
Split peas.............	34.5	32.6	35.8	19.5	16.3	45.5
Alfalfa (ground whole)..	25.3	29.6	29.6	24.3	5.3	17.9
Potato leaf............			140	62	78	55.7
Dog food (dry type)....	60.5	65.8	73.1	41.8	31.3	42.8
Skim milk powder......	7.8	8.2	8.2	<1	8.1	99.0
Rat liver‡.............	160	162	154	6	148	96.0
" muscle‡.........	71	73	78	5	73	93.5
" kidney‡..........	82	86	87	8	79	91.0

* All samples were autoclaved at 121° for 1 hour with 50 volumes of the respective reagent.

† Does not differentiate between free nicotinamide and bound nicotinamide (coenzyme I or II) or a precursor of nicotinic acid which is unavailable to the organism until after acid hydrolysis.

‡ Analyses presented on fresh weight basis. All other analyses are on the dry weight basis.

The most striking results (Table II) are the differences observed between the nicotinamide content of animal and plant products. The activity in animal products is due almost entirely to nicotinamide, whereas in plant products there is considerable variation in nicotinamide concentration,

although in most plant materials the major amount of activity appears as niacin.

<div align="center">DISCUSSION</div>

It should not be concluded that the nicotinamide as measured with *Leuconostoc mesenteroides* is all "free amide," since this method does not distinguish between the free amide and that bound as coenzyme. It is interesting to note that the potato leaf and wheat germ, both sites of great or potentially great metabolic activity, contain the largest amount of nicotinamide. This probably is indicative of the importance of the rôle of coenzymes I and II in the active metabolic processes. The apparently high nicotinamide content of white potatoes is probably due to the high percentage of nicotinic acid precursor in this product (8) rather than to nicotinamide; this is also true of wheat bran and wheat middlings.

It is interesting to speculate that the large amount of niacinamide in animal tissues indicates this compound, as opposed to niacin, as a prerequisite for coenzymes I and II needed in respiration, whereas the respiration process in plant tissues and seeds utilizes niacin either for a reservoir to be converted to the amide as the respiration process is stimulated or for some other as yet undetermined metabolic function. It is interesting to consider the fact that the part of the plant having the least active metabolism, namely the bran or hull of the seed, has the greatest concentration of niacin, whereas the germ, the seat of potentially great metabolic activity, has the largest percentage concentration of nicotinamide.

<div align="center">SUMMARY</div>

The conditions for hydrolyzing nicotinamide to nicotinic acid and its measurement by *Leuconostoc mesenteroides* have been evaluated. The use of 1 N HCl for hydrolysis proved to be most satisfactory.

Selected samples of plant and animal materials were analyzed for nicotinamide by the use of the differential assay with *Leuconostoc mesenteroides* and it was found that animal tissues contain nearly all of the total niacin activity as niacinamide, while plant tissues contain much less and a more variable amount of the total activity as niacinamide.

<div align="center">BIBLIOGRAPHY</div>

1. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139,** 675 (1941).
2. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15,** 471 (1943).
3. Teply, L. J., Strong, F. M., and Elvehjem, C. A., *J. Nutr.*, **23,** 417 (1942).
4. Hale, E. B., Davis, G. K., and Baldwin, H. R., *J. Biol. Chem.*, **146,** 565 (1942).
5. Barton-Wright, E. C., *Biochem. J.*, **38,** 314 (1944).
6. Rosenberg, H. R., Chemistry and physiology of the vitamins, New York (1942).
7. Johnson, B. C., *J. Biol. Chem.*, **159,** 227 (1945).
8. Krehl, W. A., and Strong, F. M., *J. Biol. Chem.*, **156,** 1 (1944).

AN ELECTROPHORETIC STUDY OF THE SALT FRACTIONATION
OF YEAST EXTRACTS*

By KURT G. STERN, ARNOLD H. SCHEIN, AND JAMES S. WALLERSTEIN

(*From the Department of Chemistry, Polytechnic Institute, Brooklyn, the Department of Physiology and Biochemistry, New York Medical College, and the Overly Biochemical Research Foundation, New York*)

The present experiments were undertaken after completion of the electrophoretic analysis of crude maceration extracts from dried top and bottom yeasts, which formed the subject of an earlier publication (1). It was found that extracts of this type, commonly designated as *Lebedev juice*, contain a number of electrochemically different colloidal components. Upon charting the "molecular spectrum" with the aid of the Tiselius electrophoresis apparatus, marked quantitative differences were found to exist in the diagrams obtained with the extracts from various yeast types. Furthermore, the electrophoretic patterns of undialyzed maceration extracts appear to be more complex than those of the dialyzed solutions.

Yeast maceration extracts are known to contain a large number of substances, both of protein and non-protein character. Thus, in addition to the complete enzyme system of alcoholic fermentation (*zymase complex*), hydrolytic enzymes, dehydrogenases, flavoproteins, and hemoproteins (*cf.* (2)), as well as "inert" soluble proteins and polysaccharide (yeast gum), have been demonstrated in such preparations. Perhaps the most widely employed technique for isolating biologically active proteins from such sources as yeast or tissue extracts is the fractional salting-out method in which are utilized the differences in solubility of individual proteins in solutions of ammonium sulfate and other neutral salts of varying degrees of saturation. Frequently the salting-out method is combined with isoelectric precipitation procedures or with a fractionation involving the use of water-miscible organic solvents, especially alcohol and acetone. Although ultracentrifugal and electrophoretic techniques of separating biological colloid mixtures possess, as a rule, a higher degree of specificity and resolving power than chemical fractionation methods, the latter still remain the only ones practical on a larger scale owing to the limited capacity of the physical tools mentioned above. Their chief application at the

present time consists in their use in controlling the results of chemical fractionation procedures. For this reason it was thought of interest to follow the ammonium sulfate fractionation of bottom yeast maceration extracts by electrophoretic analysis of the system at various stages of the process. This affords a correlation of chemical and electrochemical data of a type which has been found useful in the field of serum and tissue proteins (*cf.* (3)).

<div align="center">EXPERIMENTAL</div>

Materials and Preparation of Yeast Extracts—10 pound batches of washed and pressed brewers' bottom yeast were obtained through the courtesy of the Krueger Brewing Company of Newark, New Jersey. The yeast was driven through a coarse sieve and then dried in layers of about 1 cm. in height over a period of several days at 30°. It has been established that the cell membranes are ruptured by partial autolysis under these conditions. This explains why aqueous maceration extracts from such dried yeast preparations contain the enzyme system of alcoholic fermentation (zymase) in soluble and usually active form, in contrast to similar extracts obtained from fresh or from more rapidly dried yeast. The dried yeast was stored at room temperature. At the time when the present experiments were performed, the dried yeast preparations were 6 to 8 months old. Lebedev juice is usually prepared from dried yeast by maceration with tap water at 37°. In the present work, neutral phosphate buffer was substituted for the water, in accordance with the recent experiences of other investigators (4) who found that the presence of phosphate ions is beneficial for the enzymatic and fermentative activity of yeast extracts. Furthermore, the method of salt fractionation here adopted was originally carried out on such phosphate extracts from yeast (see below).

Ammonium Sulfate Fractionation—The relative paucity of information on the fundamental properties of the proteins present in yeast extracts made the choice of a specific fractionation procedure somewhat difficult. It was finally decided to follow the method of ammonium sulfate fractionation employed by Green, Herbert, and Subrahmanyan (5) in their attempt at isolating yeast carboxylase. Ammonium sulfate has previously been used as a stabilizing and extracting agent for this enzyme (6). Inasmuch as the aim of the present investigation was the electrophoretic analysis of the various components obtainable from yeast extracts by salt fractionation rather than the isolation, in pure form, of a specific constituent, such as carboxylase, all fractions were examined in the Tiselius apparatus without regard to their enzymatic activity.

Electrophoresis Technique—The technique of electrophoretic analysis of the various extracts and fractions obtained in this study and the Tiselius

apparatus employed were the same as in the preceding report (1). Inasmuch as it was desirable to establish the experimental conditions most likely to yield reproducible results, all solutions were equilibrated against the supernatant buffer by dialysis through cellophane at low temperature, even though this might cause a loss of enzymatic activity through the diffusion of coenzymes and low molecular activators. The buffer chosen for the majority of the electrophoresis experiments was prepared by mixing 94 parts of secondary and 6 parts of primary sodium phosphate; the molarity with regard to total phosphate was 0.05, the ionic strength was approximately 0.12, and the pH close to 7.3. In order to ascertain that the protein solutions had been properly equilibrated against the buffer, the conductivity as well as the hydrion concentration of all solutions was determined after dialysis. The non-dialyzable, presumably protein nitrogen of the solutions studied varied from 2.3 to 0.15 mg. per ml. It decreased with the progress of the fractionation, although the ammonium sulfate precipitates were redissolved for examination in progressively smaller volumes of solvent.

The potential gradients employed in the electrophoresis experiments ranged from $F = 2.6$ to 3.6 volts per cm. The ground surfaces of the electrophoresis cell were lubricated with the vaseline-paraffin oil mixture recommended by Tiselius instead of the Celloseal grease employed in the previous experiments (1). The diagrams were recorded at a photographic magnification factor of 1.074[1] and were further enlarged 3.45 times by projection for tracing purposes and subsequent planimetry.

The solutions studied in this work were, as a rule, clear; they were either colorless or yellow. In order to prevent a shading of the plates due to the color which tends to be accentuated by the spectral distribution of the light source employed (General Electric mercury high pressure burner, type H-4), Wratten panchromatic plates, or Eastman trichromatic X-plates, type B, were used for the recording of the electrophoresis patterns by Longsworth's schlieren scanning technique (7).

Observations and Results

In the course of this work a total of three fractionations was carried out, according to the scheme shown in the accompanying flow sheet.

Inasmuch as all fractionations were performed in a similar manner, only one of them, *viz.* Preparation 2, will be described in some detail.

Step I—500 gm. of dried Krueger's bottom yeast, Batch 12a, were suspended in 1500 ml. of 0.066 M phosphate buffer of pH 7.2. The suspension

---

[1] The mobility data reported in the preceding paper (1) were computed on the basis of a photographic magnification factor of 1.37. Inasmuch as a recalibration of the apparatus revealed that the actual value is 1.074, the mobilities quoted in the previous publication were too high by a factor of 1.27.

Flow Sheet of Yeast Extract Fractionation

Dried bottom yeast

Macerated

Lebedev juice, Fraction I, Fig. 1

Treated with calcium acetate + phosphate buffer, pH 7, centrifuged

Supernatant, Fraction II — Ppt., discarded

Brought to 0.5 ammonium sulfate saturation

Filtrate, Fraction IIIb, Fig. 2 (discarded) — Ppt., Fraction IIIa

Dissolved in citrate buffer, pH 6, filtered

Solution of Fraction IIIa, Fig. 3

Brought to 0.5 ammonium sulfate saturation

Filtrate, Fraction IVb (discarded) — Ppt., Fraction IVa

Dissolved in citrate buffer, pH 6, filtered

Solution of Fraction IVa

Brought to 0.26 ammonium sulfate saturation

Ppt., Subfraction 1 Dissolved in phosphate buffer, pH 7.7, filtered — Filtrate

Solution of Subfraction 1, Fig. 4 (discarded)

Brought to 0.5 ammonium sulfate saturation, filtered

Filtrate — Ppt., Subfraction 2

Brought to 0.73 ammonium sulfate saturation

Dissolved in phosphate buffer, pH 7.7, filtered

Filtrate (discarded)

Solution of Subfraction 2, Fig. 5

Ppt., Subfraction 3

Dissolved in citrate buffer, pH 6, brought to 0.25 ammonium sulfate saturation

Solution of Subfraction 3, Fig. 6

Brought to 0.67 ammonium sulfate saturation in 2 stages; no Subfraction 3a

Ppt., Subfraction 3b

Dissolved in citrate buffer and brought to 0.25 ammonium sulfate saturation

Solution of Subfraction 3b, Fig. 7

Brought to 0.66 ammonium sulfate saturation in 2 steps; no Subfraction 3b$_1$ obtained

Filtrate

Brought to 0.83 ammonium sulfate saturation

Filtrate (discarded)

Ppt., Subfraction 3b$_3$

Dissolved in phosphate, pH 7.7

Solution of Subfraction 3b$_3$, Fig. 11

Filtrate

Brought to 0.82 ammonium sulfate saturation

Filtrate (discarded)

Ppt., Subfraction 3e

Dissolved in phosphate, pH 7.7

Solution of Subfraction 3e, Fig. 8

Brought to full ammonium sulfate saturation

Ppt., Subfraction 3b$_2$

Dissolved in citrate, pH 6

Solution of Subfraction 3b$_2$, Fig. 9

Filtrate (discarded)

Ppt., recovered Subfraction 3b$_2$

Dissolved in phosphate, pH 7.7

Solution of recovered Subfraction 3b$_2$, Fig. 10

was stirred mechanically for 1 hour at 36–37° on the water bath.   2000 ml. of tap water of room temperature were added and the diluted suspension was placed for 1 hour in the refrigerator.   It was then centrifuged for 20 minutes at 2000 R.P.M.   The supernatant Lebedev juice (Fraction I) which was decanted from the sediment had a volume of 2570 ml. and a nitrogen content, as determined by Pregl's micro modification of the Kjeldahl method, of 2.3 mg. per ml.   An aliquot of 20 ml. was dialyzed in the refrigerator against 2 liters of 0.05 M phosphate buffer, pH 7.7, and examined in the electrophoresis apparatus.   The diagram obtained in this way (Experiment 317) is reproduced in Fig. 1.   It discloses the presence of five more or less resolved components of a mobility ranging from -0.74 to -6.0×10^{-5} cm.^{2} per second per volt for the descending boundaries. The major amount of material was contained in the fraction of intermediate mobility (see Table I).   The pattern here obtained resembles that yielded by a similar extract in the previous publication ((1), Fig. 9), except that

←——DESCENDING   ASCENDING——→
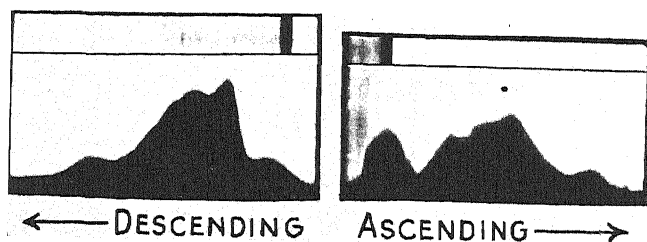
FIG. 1. Electrophoretic diagram of crude, dialyzed Lebedev juice from Krueger's bottom yeast (Batch 12a), Fraction I.

the resolution of the intermediate fraction is greater in the present instance. The nomenclature employed in the present paper with regard to the designation of the various electrophoretic components is the same as that used previously (1); we do not wish to imply, however, that components bearing the same designation are necessarily identical.   It is quite possible that the quasistationary peaks (designated as γ component) are in part buffer and protein concentration gradients (boundary anomalies), similar to the δ and ε maxima observed in the electrophoretic diagram of blood serum (cf. (8)).

Step II—The remainder of the Lebedev juice (2550 ml.) was mixed with 400 ml. of 0.5 M phosphate buffer, pH 7.0, and then with 200 ml. of M calcium acetate solution.   The heavy precipitate, consisting largely of calcium phosphate, was centrifuged off (10 minutes at 2000 R.P.M.) and discarded.   The volume of the clear, yellow supernatant solution (Fraction II) amounted to 2810 ml.; the nitrogen content was 2.06 mg. per ml., indicating that little if any protein had been adsorbed on the calcium phosphate.   Of this solution, 20 ml. were equilibrated against 0.05 M

phosphate buffer, pH 7.7, prior to examination in the Tiselius apparatus. The pattern recorded (Experiment 316) was very similar to that obtained from the original Lebedev juice (see Fig. 1). The differences in apparent mobility of the various components (see Table I) compared with those present in the Lebedev juice are possibly due to a slight hydrostatic shift which may have occurred during the experiment in the Tiselius cell (note the apparent cathodic mobility of the γ-boundary).

Step III—To the balance of the supernatant fluid remaining after the calcium phosphate precipitation (2790 ml.) there were added 964 gm. of purified ammonium sulfate to bring the solution to 0.5 saturation. A precipitate formed immediately upon addition of the salt. The suspension was kept in the refrigerator overnight and was then filtered by suction on a large Büchner funnel through filter paper covered with a 3 mm. layer of

←——DESCENDING   ASCENDING——→
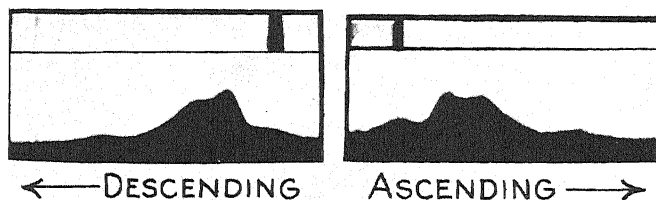
FIG. 2. Electrophoretic diagram of Fraction IIIb

Hyflo Super-Cel. The clear, yellow filtrate (Fraction IIIb), upon examination (Experiment 318) in the Tiselius apparatus after dialysis against phosphate buffer, showed a composition somewhat resembling that of the preceding solutions but the peaks visible in the electrophoretic diagram (Fig. 2) enclose appreciably smaller areas, in keeping with the lower total nitrogen content (0.71 mg. of N per ml.). The rest of the filtrate was discarded. The filter cake remaining after removing as much of the filtrate as possible by pressing was suspended in 750 ml. of 0.04 M citrate buffer of pH 6.0. A slight turbidity was removed by filtration and an aliquot of the clear, yellow solution (Fraction IIIa) was subjected to electrophoretic analysis (Experiment 319). The resulting diagram (Fig. 3) showed the presence of three to four components with mobilities ranging from -0.1 to -6.27×10^{-5} cm.^{2} per second per volt (Table I). The nitrogen content of the solution was 1.32 mg. per ml.

Step IV—The remainder of the solution was brought to 0.5 saturation by adding 305 gm. of ammonium sulfate to approximately 800 ml. of the solution. After storage overnight in the refrigerator, the suspension was filtered by suction through Hyflo Super-Cel. The clear, greenish yellow filtrate (Fraction IVb), which contained only a trace of protein, was dis-

carded. The precipitate remaining on the filter was dissolved in 500 ml. of 0.04 M citrate buffer, pH 6. A small amount of insoluble material was removed by filtration and an aliquot of the clear filtrate (Fraction IVa) was examined in the Tiselius apparatus (Experiment 320). The resulting pattern was very similar to that recorded with Fraction IIIa (see Fig. 3), as would be expected from the fact that most of the protein material had been recovered in this fraction. Fraction IVa contained 1.56 mg. of N per ml. or 87 per cent of the total nitrogen present in Fraction IIIa.

Step V—The material, contained in Fraction IVa, representing proteins precipitable at 0.5 saturation with ammonium sulfate, was now further fractionated as follows: To 530 ml. of solution of Fraction IVa were added 185 ml. of saturated ammonium sulfate solution, yielding a 0.26 saturated solution. After standing in the cold overnight, the suspension was filtered
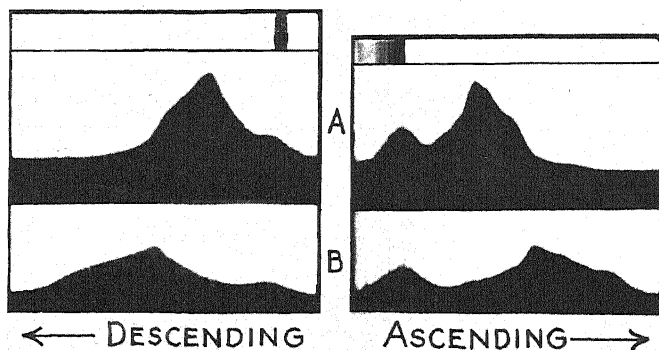
FIG. 3. Electrophoretic diagram of Fraction IIIa

through Hyflo Super-Cel. The precipitate remaining on the filter was dissolved in 100 ml. of 0.05 M phosphate buffer, pH 7.7, and filtered to obtain a clear solution. According to electrophoretic analysis (Experiment 321, Fig. 4) this fraction, designated as *Subfraction 1*, contained two major components (α and β) of a mobility of -6 and -4×10^{-5} cm.^{2} per second per volt respectively. The descending patterns revealed, in addition, the presence of a trace of a highly mobile material (u $= -8.7$). The nitrogen content amounted to 0.4 mg. per ml. To the filtrate remaining after precipitating *Subfraction 1* at 0.26 saturation with ammonium sulfate there were added 350 ml. of saturated ammonium sulfate solution, raising the salt concentration to 0.5 saturation. A bulky precipitate formed which was filtered off as usual and dissolved in 300 ml. of 0.05 M phosphate buffer of pH 7.7. After clarification, an aliquot was examined in the Tiselius apparatus (Experiment 322) and found to contain essen-

tially material of intermediate mobility (β components), as shown in Fig. 5 and Table I.

The solution contained 1.64 mg. of N per ml.; it was designated *Subfraction 2*. The filtrate remaining after the removal of *Subfraction 2* (volume 990 ml.) was brought to 0.73 saturation by the addition of 870 ml. of saturated ammonium sulfate solution. The resulting precipitate was dissolved in a mixture of 50 ml. of 0.1 M citrate buffer, pH 6, 50 ml. of
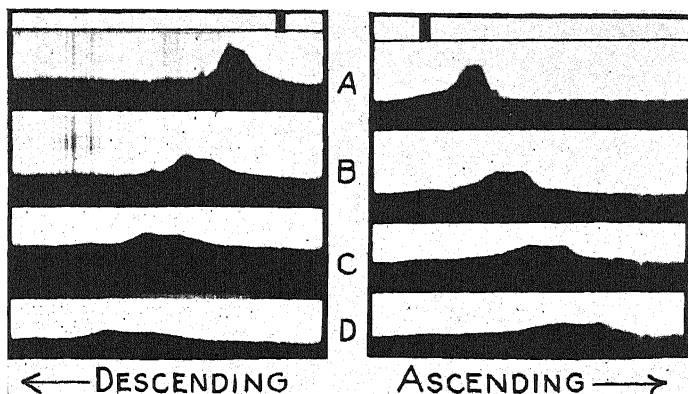
FIG. 4. Electrophoretic diagram of Subfraction 1
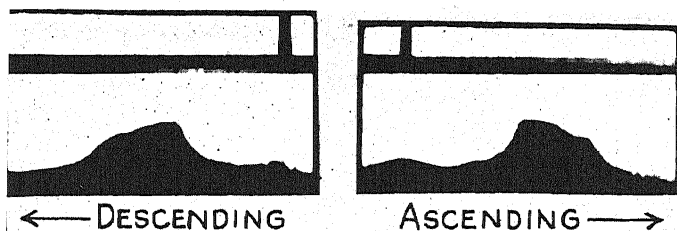
FIG. 5. Electrophoretic diagram of Subfraction 2

saturated ammonium sulfate solution, and 100 ml. of distilled water, corresponding to 0.25 saturation with regard to ammonium sulfate. An aliquot of the filtered solution, called *Subfraction 3*, was subjected to electrophoretic study (Experiment 323). The diagram (Fig. 6) showed a relatively large amount of an initially well defined fraction of a mean mobility of -4.17×10^{-5} cm.^{2} per second per volt and a smaller amount of a component of a mean mobility of -6.3. As in the instance of most other components here observed, the former showed a considerable tendency to spread in the later part of the experiment. The nitrogen content of the solution was 0.49 mg. per ml. The filtrate obtained after precipitating

Subfraction 3 gave a negative test for protein with sulfosalicylic acid and hence was discarded. The nitrogen content of all three subfractions amounted to 73 per cent of the nitrogen content of Fraction IVa; the bulk of the nitrogen, *viz.* 57 per cent, was contained in *Subfraction 2*, while *Subfraction 1* contained about 5 per cent and *Subfraction 3* represented approximately 11 per cent of the nitrogen of Fraction IVa.

Step VI—Subfraction 3, which, according to Green *et al.* (5), would be expected to contain the bulk of the yeast carboxylase, was further fractionated in the following manner. The remainder of *Subfraction 3*,
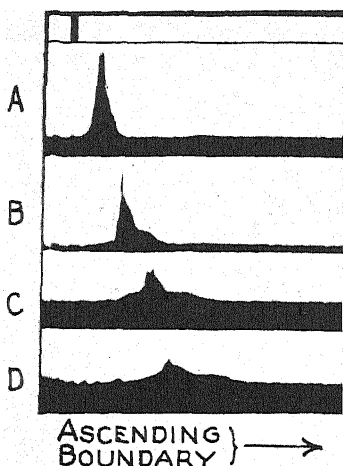
Fig. 6. Electrophoretic diagram of Subfraction 3.
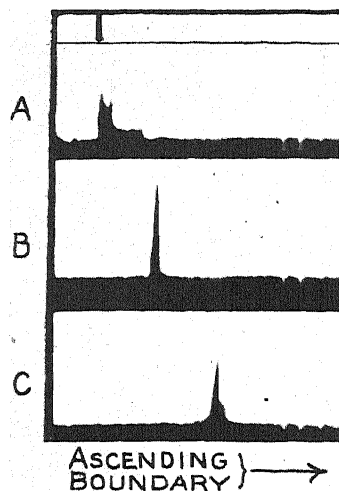
Fig. 7. Electrophoretic diagram of Subfraction 3b.

amounting to 180 ml., was first brought to 0.5 saturation of ammonium sulfate by the addition of 80 ml. of saturated salt solution. Since no precipitate, corresponding to Green's *Subfraction 3a*, formed at this stage, an additional 143 ml. of saturated ammonium sulfate solution were added, raising the salt concentration to 0.67 saturation. The resulting precipitate was dissolved in 40 ml. of 0.1 M citrate buffer, pH 6, 40 ml. of saturated ammonium sulfate solution, and 80 ml. of water. Of this solution, representing *Subfraction 3b*, 15 ml. were dialyzed against phosphate buffer and submitted to electrophoretic examination (Experiment 324). The diagram (Fig. 7) showed essentially one, very sharp boundary migrating at pH 7.32 at a mobility of -5.7×10^{-5} cm.^{2} per second per volt in the ascending and -6.0 in the descending limb of the apparatus. This boundary, which showed only a slight tendency to spread during electrophoresis, would correspond to an α component on the basis of its mobility (see Table I).

The filtrate obtained after the removal of *Subfraction 3b* was brought to 0.82 saturation by the addition of 292 ml. of saturated ammonium sulfate solution to 380 ml. of protein solution. The precipitate which now formed was dissolved in 20 ml. of 0.05 M phosphate buffer, pH 7.7, clarified by filtration (*Subfraction 3c*), and studied in the Tiselius apparatus (Experiment 325). According to the pattern recorded of this fraction (Fig. 8) several components of mobilities ranging from -3.0 to -7.1×10^{-5} cm.^{2} per second per volt (for the ascending boundaries) were present in this subfraction. 82 per cent of the material was of β mobility (see Table I). All boundaries showed a considerable tendency to spread during electrophoresis, in contrast to the boundary observed in *Subfraction 3b*. The nitrogen content of *Subfraction 3c* was 0.5 mg. per ml.
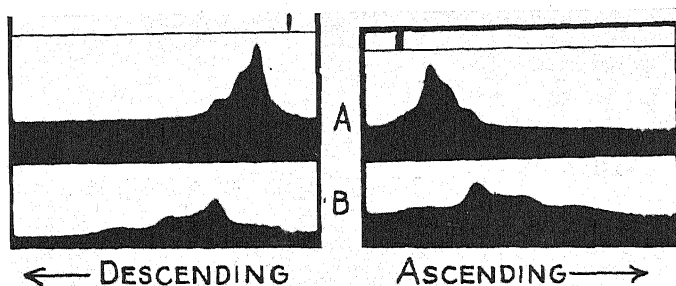
Fig. 8. Electrophoretic diagram of Subfraction 3c

Step VII—Subfraction 3b, which according to Green contains the enzyme carboxylase, was further fractionated as follows: To 140 ml. of *Subfraction 3b* there were added 57.5 ml. of saturated ammonium sulfate solution, bringing the solution to about 0.5 saturation. Since no precipitate, corresponding to Green's *Subfraction 3b₁*, appeared at this point, 115 ml. of saturated ammonium sulfate solution were added, raising the concentration to 0.66 saturation. The precipitate which formed was dissolved in 30 ml. of 0.04 M citrate buffer, pH 6; the solution was clarified by filtration (*Subfraction 3b₂*). One-half of this solution was first used for electrophoretic analysis (Fig. 9, Experiment 326). Only one boundary of a mobility of -5.4×10^{-5} cm.^{2} per second per volt was recorded on the ascending as well as the descending side (see Fig. 10). This boundary, which exhibited the mobility of an α-protein fraction, showed a considerably greater tendency to spread during electrophoresis than did the single boundary observed in *Subfraction 3b* (compare with Fig. 7). The nitrogen content of this solution was less than 0.68 mg. per ml. The material was recovered from the electrophoresis cell, combined with the remainder of *Subfraction 3b₂*, and was precipitated at full saturation with ammonium sulfate. The

precipitate was redissolved in 7 ml. of 0.05 M phosphate buffer, pH 7.7, and the resultant clear, yellow solution was equilibrated against 2 liters of phosphate buffer by dialysis. When this *reconcentrated Subfraction 3b₂* was examined in the Tiselius apparatus, the appearance of the (Fig. 10, Experiment 329) electrophoretic pattern in the ascending limb was the same as that of the original *Subfraction 3b₂*, except for the presence of a
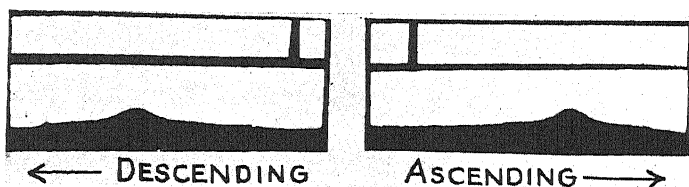
Fig. 9. Electrophoretic diagram of Subfraction 3b₂
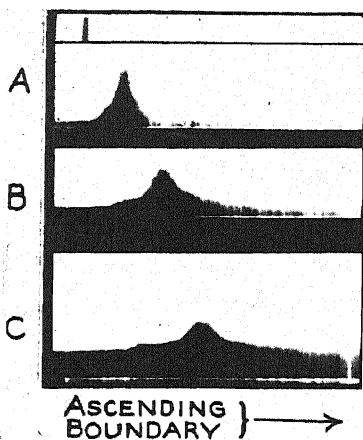
Fig. 10   Fig. 11

Fig. 10. Electrophoretic diagram of Subfraction 3b₂, concentrated by ammonium sulfate precipitation.
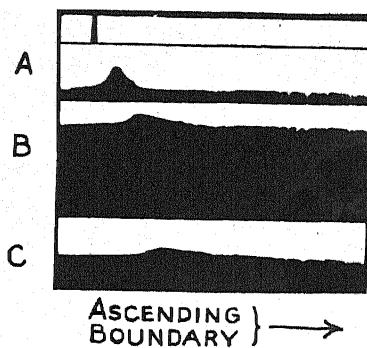
Fig. 11. Electrophoretic diagram of Subfraction 3b₃.

trace of a component of lower mobility. The mobility of the main component amounted to -4.4×10^{-5} cm.² per second per volt, which is somewhat lower than that of the corresponding material in the original subfraction. This might indicate a modification of the protein under the influence of high salt concentration.

The filtrate from *Subfraction 3b₂* was brought to 0.83 saturation by adding 330 ml. of saturated ammonium sulfate solution to 330 ml. of the filtrate. The precipitate which formed was dissolved in 11 ml. of 0.05 M phosphate

buffer, pH 7.7, clarified by filtration (*Subfraction 3b₃*), and subjected to electrophoretic examination after equilibration against the same buffer (Experiment 327, Fig. 11). As would be expected from the low nitrogen content (0.15 mg. per ml.), only a very low peak was observed in this subfraction (Fig. 11). The mobility of this material, *viz.* -3.6×10^{-5} cm.^{2} per second per volt, was definitely lower than that of the boundary recorded for *Subfraction 3b₂*. The mobility values as well as estimates of the relative concentrations of the various electrophoretic components observed in these experiments are given in Table I.

No claim is made with regard to the precision of these data. They are listed chiefly as an aid in labeling and tracing the individual components.

DISCUSSION

The *optical* resolution of crude dialyzed yeast maceration extracts into individual components by the moving boundary method of electrophoresis is considerably less complete than that of the proteins in blood serum, for example. Under these conditions a successful *mechanical* separation of the individual colloids present in these extracts by preparative electrophoresis would not seem to be very promising. However, the present experiments show that electrophoretic analysis is useful in determining the success of chemical fractionation procedures at every step of the process. As judged by the results obtained with this physical tool, half saturation of crude yeast extracts with ammonium sulfate does not produce a fractionation as decisive as that of blood serum into globulin and albumin components by the same means. Especially pronounced in the case of yeast proteins is the tendency of coprecipitation of components of high and intermediate ammonium sulfate solubility in the early stages of the fractionation procedure. From the point of view of extent of separation, Step II (calcium phosphate precipitation) appears to be the least efficient and Step IV (fractional ammonium sulfate precipitation) the most effective operation. It is also noteworthy that fractions of similar electrophoretic mobility, present in the crude extracts, exhibit significant differences with respect to their solubility in ammonium sulfate. This is similar to the finding of Tiselius (3) that the individual globulin components of blood serum contain fractions of different solubility in spite of closely similar electrophoretic mobility.

As may be seen from the diagrams reproduced in this paper, the procedure of Green *et al.*, employed in the purification of yeast carboxylase (5), leads to the isolation of a protein component which has a well defined mobility (5.4×10^{-5} unit at pH 7.3) and a fair degree of electrochemical homogeneity. It should be noted, however, that Green *et al.* state that their enzyme preparation of the highest activity ratio which they were

TABLE I

Electrophoretic Analysis of Yeast Maceration Extract (Krueger's Bottom Yeast) and Fractions Prepared by Ammonium Sulfate Precipitation

Experiment No.	Fraction	Designation	pH	Boundaries*	Relative concentration of components, per cent						Electrophoretic mobility,† cm² sec.⁻¹ volt⁻¹ × 10⁵						Electrophoresis period No.
					γ	β_1	β_2 and β_3	β, total	α, total	α and β, total	γ	β_1	β_2 and β_3	β', total	α_1	α_2	
317	Lebedev juice	Fraction I	7.33	A.	21	19	53	72	8		0.10	2.12	3.94		6.48		
				D.	6	28	56	84	9		0.74	1.85	2.86		6.0		
316	Filtrate, after calcium phosphate pptn.	" II	7.23	"	5	22	64	86	9		+0.14	1.24	2.40		5.55		
319	Ppt., 0.50 ammonium sulfate saturation, dissolved and reprecipitated	" IIIa	7.35	A.	15					85	0.10			4.03	6.27		
				D.	13					85	0.60			3.75			
318	Filtrate from 0.50 saturated ammonium sulfate solution, discarded	" IIIb	7.42	A.	7	41	46	87	4			1.79	3.05		6.04		
				D.	10	34	52	86	4			1.75			6.10		
320	Ppt., 0.50 ammonium sulfate saturation, dissolved and fractionated	" IVa	7.40	A.	11				89		+0.42			3.67			
				D.	6				95		+0.53			3.59			
321	Ppt., 0.26 ammonium sulfate saturation, discarded	Subfraction 1	7.30	A.										3.9	5.93		2
				D.										4.15	5.98	8.24	3
				A.										4.0	6.07		
				D.											6.16	8.80	
322	Ppt., 0.50 ammonium sulfate saturation, discarded	" 2	7.18	A.	4			96			0.16			4.09			
				D.	4			96			0.40			3.82			
323	Ppt., 0.73 ammonium sulfate saturation, dissolved and refractionated	" 3	7.23	A.				72	28					4.22	6.42		3
				"				68	28					4.12	6.12		4

No.	Description	Subfraction		A./D.	Peak								Count
324	Ppt., 0.67 ammonium sulfate saturation, dissolved and again fractionated	Subfraction 3b	7.32	A.	Multiple peak, incompletely resolved							5.75	2
				D.								6.0	
325	Ppt., 0.82 ammonium sulfate saturation, discarded	3c	7.44	A.		43	39	82	17	3.05	4.90	7.15	
				D.		54	27	81	18	3.17	5.06	7.28	
326	Ppt., twice refractionated with 0.67 saturated ammonium sulfate	3b₂	7.28	A.	Single component, very low concentration							5.36	
				D.								5.44	
327	Ppt. from filtrate of Experiment 326, 0.82 ammonium sulfate saturation	3b₃	7.60	"	Multiple peak, incompletely resolved							3.57	2
329	Ppt. (altered?), 3 × refractionated, 0.67 ammonium sulfate saturation		7.52	"								4.43	

* A. = ascending; D. = descending.

† All mobilities listed in the table are anodic mobilities and, hence, negative in sign unless otherwise noted.

able to obtain was not homogeneous. As is shown in this paper (see Figs. 9 and 10), the reprecipitation by ammonium sulfate of the protein isolated by Green's method produces a change in electrophoretic behavior and pattern. Upon examination in the analytical ultracentrifuge this material was found to be polydisperse. It would appear, therefore, that in highly purified form this protein shows a greater sensitivity towards high salt concentration than in the crude extract, an experience not uncommon in the field of biologically active proteins. It would be of interest to correlate the electrophoretic components here observed with proteins previously isolated from yeast; e.g., the crystalline proteins described recently by Kunitz and McDonald (9).

SUMMARY

The fractionation of the proteins present in yeast maceration extract (Lebedev juice), essentially by ammonium sulfate precipitation, has been followed by electrophoretic analysis of the various fractions in the Tiselius apparatus.

It could be demonstrated that the procedure employed by Green and his associates in the purification of yeast carboxylase leads to the isolation of a fairly well characterized protein fraction. This protein occurs in the crude extracts only in small amounts and it has been possible to follow the elimination of large amounts of ballast proteins and other colloids by controlling the chemical fractionation procedures by electrophoretic analysis at each stage of the process. No attempt has been made to correlate specific biological activity with the electrochemical properties of the individual components. Data are presented for the electrophoretic mobilities and approximate relative concentrations of the various colloidal components of yeast maceration extract.

BIBLIOGRAPHY

1. Stern, K. G., *J. Biol. Chem.*, **152**, 345 (1944).
2. Oppenheimer, C., and Stern, K. G., Biological oxidation, The Hague (1939).
3. Tiselius, A., *Biochem. J.*, **31**, 1464 (1937).
4. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1**, 191 (1942).
5. Green, D. E., Herbert, D., and Subrahmanyan, V., *J. Biol. Chem.*, **138**, 327 (1941).
6. Melnick, J. L., and Stern, K. G., *Enzymologia*, **8**, 129 (1940).
7. Longsworth, L. G., *J. Am. Chem. Soc.*, **61**, 529 (1939).
8. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, **63**, 119 (p. 126) (1939).
9. Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, **29**, 143 (1946).

ELECTROLYTIC SEPARATION OF BASIC, NEUTRAL, AND ACIDIC AMINO ACIDS IN PROTEIN HYDROLYSATES*

By ERIK SPERBER

(From Wenner-Gren's Institute for Experimental Biology, Stockholm, Sweden)

For many years it has been obvious that separation of the different classes of amino acids might be achieved by electrophoresis. Theoretically it ought to be possible to obtain the monoaminodicarboxylic acids in the anodic compartment, the monoaminomonocarboxylic acids in the middle compartment, and the dibasic monocarboxylic acids in the cathodic compartment of a three cell apparatus. In fact such a separation is claimed to have been performed with some degree of exactitude by Theorell and Åkeson (1), who worked on a micro scale. The main difficulty of the method is to keep the pH of the middle cell of the apparatus constant at about 6. For this purpose the membranes between the different compartments must have certain properties, not easily realized. Theorell and Åkeson used parchment paper and parchment freed from all but the epidermal parts by an operation not possible to carry out with areas larger than a few sq. cm.

Another difficulty, which, however, is easier to overcome, is that glutamic and aspartic acids are attacked by any chlorine that may be evolved in the anodic compartment following the discharge of chloride ions. Generally the solutions must be freed of these ions before treatment in the electrolysis apparatus.

If the ideal membrane combination has not been found, the pH of the middle compartment changes, causing the neutral amino acids to assume a net charge and hence to migrate to the anodic or cathodic compartment. Generally the pH turns acid, so that the neutral amino acids migrate towards the cathode. The addition of a buffer substance capable of keeping the pH between proper limits would prevent this and would make the whole process practicable, especially if the membranes were impermeable to the substance in question.

In recent years such substances have been commercially produced; namely the anion-exchanging synthetic resins of the type of Amberlite IR-4. These substances have already been used for the purpose of separating amino acids by adsorption, for both analytical (Freudenberg *et al.* (2)) and preparative (Cannan (3)) purposes.

In reviewing the method theoretically, Cannan found that the presence of hexone bases interfered with the quantitative adsorption of the dicarboxylic acids, a difficulty which he circumvented by using large amounts of hydrochloric acid to be adsorbed simultaneously with the amino acids or by first freeing the solution from the major part of the hexone bases with phosphotungstic acid. It is obvious that if, as is the case in yeast hydrolysates, the solution contains significant amounts of other positive ions such as K·, etc., the latter procedure is of limited value. In such cases it might be expected that electrophoresis would prove a useful method of freeing the solution from all positively charged ions.

The present author has therefore attempted to combine the two procedures, adsorption according to Cannan, and electrophoresis, in order to obtain an easy method for the separation of the different classes of amino acids.

Procedure

A few changes were made in the construction of the electrophoresis apparatus of Albanese (4), a cooling coil being inserted into the central compartment.

The procedure worked out was the following. A protein hydrolysate in HCl was partially freed from the acid by repeated evaporation to dryness *in vacuo*, dissolved in water, and filtered to remove humin. The solution was stirred for a few hours with Amberlite IR-4 and transferred to the middle cell of an electrophoresis apparatus together with some fresh Amberlite. In all cases the catholyte consisted initially of distilled water and the anolyte of a dilute sulfuric acid solution.

The time-current curve followed the course described by Albanese; the current was not reduced to insignificant values even after a very long time. This was due to the sample of resin used, with which the pH of the solution could not be maintained at a value higher than about 4.5 instead of the ideal value of pH 5.5 to 6.0, which was originally intended. The catholyte solution was therefore neutralized and submitted to the same procedure once more and in some cases a third time.

The pooled samples of Amberlite from the initial treatment and the first electrophoresis were treated with strong HCl to elute the monoaminodicarboxylic acids, which were then isolated or estimated in the usual manner.

It was found that only insignificant amounts of nitrogen migrated into the anode solution under these circumstances (*e.g*, in an experiment with yeast hydrolysate 0.5 mg. out of 1800 mg.). Consequently the anolytes were discarded in all cases.

Results

As an example of results obtained with the above procedure the following experiment may be cited, carried out with a synthetic hydrolysate containing, in about 300 ml. of solution, arginine (43.7 mg. of N), histidine (10.1 mg. of N), glycine (106.8 mg. of N), and glutamic acid (51.4 mg. of N). To this were added some ammonium sulfate (about 40 mg. of N), potassium phosphate, and invert sugar. After acidification with HCl, the solution was treated according to the above procedure with two electrophoreses. After the separation the acid fraction contained 49.5 mg. of N organically bound (calculated, 51.4 mg.), the neutral fraction 112.0 mg. (calculated, 106.8 mg.), and the basic fraction 51.0 mg. (calculated, 53.8 mg.). The analysis was carried out in less than 2 days. It seems very probable that still better results might have been achieved if the neutral solution had been treated once more with Amberlite and subsequently subjected to a third electrolysis. The procedure led to practically no loss of nitrogen, which is especially valuable if the hydrolysate fractions are later to be analyzed for the different amino acids.

An estimation of arginine in the "basic" solution of a similar experiment revealed that of 62.4 mg. of arginine nitrogen 60.2 mg. could be isolated in the form of arginine monoflavianate, a loss of about 3 per cent.

SUMMARY

The pH changes in ionophoresis of amino acid solutions can be overcome by adding some anion exchanger to the central compartment of a three cell apparatus. This will prevent the pH from falling lower than about 4.5, at which value practically only the hexone bases will migrate to the cathode. The glutamic and aspartic acids will largely be adsorbed to the anion exchanger (Amberlite IR-4), provided that the anode compartment is sufficiently acid. The losses of amino acids are very small during this procedure, so that it may be repeated at will, but ordinarily two runs will be sufficient to complete the separation of the amino acids into the three classes, acid, neutral, and basic.

BIBLIOGRAPHY

1. Theorell, H., and Åkeson, Å., *Ark. Kemi, Mineral. o. Geol.*, **16 A,** No. 8 (1943).
2. Freudenberg, K., Walch, H., and Molter, H., *Naturwissenschaften,* **30,** 87 (1942).
3. Cannan, R. K., *J. Biol. Chem.,* **152,** 401 (1944).
4. Albanese, A. A., *J. Biol. Chem.,* **134,** 467 (1940).

A NOTE ON *meso*-ERYTHRITOL, A METABOLIC PRODUCT OF ASPERGILLUS TERREUS

By FRANK H. STODOLA

(From the Fermentation Division, Northern Regional Research Laboratory,* Peoria, Illinois)

In a recent paper (1), we reported the formation of itatartaric acid by an ultraviolet-induced mutant of *Aspergillus terreus*. We have since found that by prolonged ether extraction of the concentrated culture liquor therein described a brown gum is obtained which, after some months standing, deposited large crystals. These crystals (3.25 gm.) could be separated from the gum on the basis of their insolubility in methanol. Decolorization with carbon and crystallization from 80 per cent alcohol gave 2.38 gm. of colorless, tetragonal crystals, m.p. 117–120°. A second crop of 0.47 gm. was obtained (m.p. 115–118°). The water solubility, neutral reaction, and negative Fehling's test for reducing sugars suggested a polyhydric alcohol. The melting point and analysis (calculated for $C_4H_{10}O_4$, 39.34 per cent C, 8.26 per cent H; found, 39.6 per cent C, 8.34 per cent H) indicated the compound to be *meso*-erythritol. Identity was established by a mixed melting point test with an authentic sample of *meso*-erythritol, m.p. 118–121°. The x-ray diffraction patterns of the natural and authentic samples were the same. The tetraacetate of the isolated product, m.p. 85–86°, was shown by the mixed melting point test and x-ray patterns to be identical with the tetraacetate of the authentic sample, m.p. 85–86°. The yield of *meso*-erythritol (2.85 gm.) was small since the amount of glucose supplied was 825 gm.

A survey of the literature shows that, although *meso*-erythritol frequently occurs in algae and lichens, there has been only one report (2) of the isolation of this sugar alcohol from mold culture liquors. The molds used in this work by Oxford and Raistrick were *Penicillium brevi compactum* Dierckx and *Penicillium cyclopium* Westling. These investigators point out that, in contrast to *meso*-erythritol, mannitol is a tissue constituent of many mold species and is sometimes found in the culture liquor in large amounts.

BIBLIOGRAPHY

1. Stodola, F. H., Friedkin, M., Moyer, A. J., and Coghill, R. D., *J. Biol. Chem.*, **161**, 739 (1945).
2. Oxford, A. E., and Raistrick, H., *Biochem. J.*, **29**, 1599 (1935).

DETERMINATION OF ASPARTIC-GLUTAMIC TRANSAMINASE IN TISSUE HOMOGENATES*

BY STANLEY R. AMES† AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

During the course of studies on the effect of pyridoxine deficiency on transaminase activity, it became evident that a simple and rapid method for the assay of transaminase in normal and abnormal tissues was necessary. In this investigation it was not essential to determine each of the constituents of the transaminating system but rather to ascertain the concentration of a single component in a rapid and exact manner.

The techniques developed by Cohen and Hekhuis (1) for the study of transamination in normal tissues have been widely accepted but the procedures are rather slow. Glutamic acid was determined according to Cohen (2, 3) by treatment with chloramine-T, followed by hydrolysis to succinic acid which could be determined by a preparation of succinic oxidase. Pyruvic acid was determined by the carboxylase method of Westerkamp (4) and α-ketoglutaric acid according to Krebs (5) by oxidation with acid permanganate to succinic acid, followed by enzymatic assay. Aspartic acid was determined by the method of Cohen (3) by measuring the carbon dioxide formation on treatment with chloramine-T.

Recently Green and coworkers (6) have developed simple and rapid methods for the study of transamination. α-Ketoglutaric acid was oxidized by means of H_2O_2 to succinic acid, which then could be determined by succinic oxidase preparations. Oxalacetic acid was determined spectrophotometrically or manometrically by measuring carbon dioxide evolution on treatment with aniline citrate (7), according to the principles developed by Ostern (8). These analytical techniques were developed specifically for purified preparations of transaminase and cannot be directly applied to the study of transaminase in whole tissue preparations.

In this contribution, a method for the determination of oxalacetic acid is developed which is applicable to the assay of aspartic-glutamic transaminase

in tissue homogenates.   Possible sources of error have been evaluated and the precision of the method has been determined.

EXPERIMENTAL

Animals were stunned by a blow on the head, decapitated, and exsanguinated.   The tissues were rapidly extirpated, rinsed with redistilled water, and immediately packed in finely cracked ice.   After cooling, bits of fat and connective tissue were removed and the tissues were blotted between moistened filter papers and rapidly weighed on a delicate torsion balance.   A homogenate of the tissue was prepared by means of the device described by Potter and Elvehjem (9) in ice-cold 0.10 M sodium potassium phosphate buffer (pH 7.3) with a prechilled tube and pestle.   This homogenate was pipetted immediately into the reaction vessels to avoid destruction of the enzyme by light, as reported by Schlenk *et al.* (10).   The dry weights of samples were determined by evaporation to constant weight in open crucibles in an electric oven at 110°.

A conventional Warburg apparatus at 37.0° was used in all experimental and analytical work and the pH was determined by means of a Beckman pH meter (glass electrode).   A summary of the components of the final reaction mixture is as follows: 0.50 ml. of 0.25 M sodium potassium phosphate buffer (pH 7.3) prepared as previously described (11), 0.50 ml. of 0.20 M sodium aspartate, 0.50 ml. of 0.10 M sodium α-ketoglutarate[1] (in one side arm), 0.50 ml. of aniline citrate reagent prepared as previously described (12) (in the second side arm), the desired amount of tissue homogenate in 0.10 M sodium potassium phosphate buffer (pH 7.3), and a sufficient quantity of this same 0.10 M buffer to yield a total volume of 3.00 ml.   After a 6 to 8 minute equilibration the stop-cocks were closed and the α-ketoglutarate was added from one side arm.   The transamination reaction was allowed to proceed for exactly 10 minutes and was stopped by adding the aniline citrate reagent from the other side arm, and mixed carefully to insure complete decomposition of oxalacetic acid.   The evolution of carbon dioxide is complete within 10 minutes.

Before applying this reaction system to tissue homogenates there are three possible sources of deviations which must be evaluated; namely, the effect of the aniline citrate reagent on tissue homogenates, the effect of tissue homogenates on the rate of disappearance of oxalacetic acid, and the determination of the carbon dioxide evolution at zero tissue concentration.

In order to determine the effect of the aniline citrate reagent on tissue homogenates, a series of determinations was made of the carbon dioxide

[1] Appreciation is expressed to Dr. R. H. Burris for a generous sample of α-ketoglutaric acid.

evolution on the addition of different quantities of tissue homogenate in the above test system, except that the α-ketoglutarate was omitted. The results of numerous determinations of this type with a number of different tissues indicated that a value of approximately 0.4 c.mm. of carbon dioxide was evolved per mg. of tissue on a wet weight basis (Fig. 1). With this figure, suitable correction must be made for each level of tissue used in the determination of oxalacetate. This correction must be applied before any attempt is made at extrapolation to determine the intercept which represents the carbon dioxide evolution at zero tissue concentration.
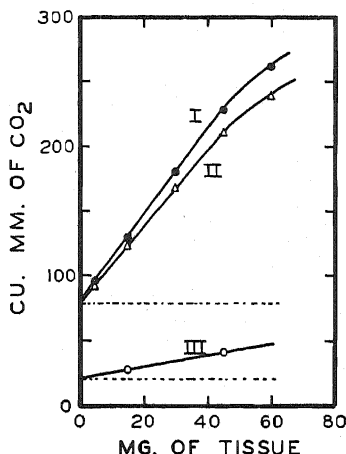
FIG. 1. Determination of transaminase in normal rat kidney. Curve I, evolution of carbon dioxide (uncorrected); Curve II, carbon dioxide evolution corrected for tissue homogenate (Curve I minus Curve III); Curve III, carbon dioxide evolution due to tissue homogenate alone (α-ketoglutarate omitted from the reaction mixture). The components of the reaction mixture are described in the text, except that a 5 per cent homogenate of normal rat kidney was used. Tissue levels are expressed on a wet weight basis.

It has been shown previously that oxalacetic acid spontaneously decomposes at 37.0°. The rate of decomposition is slow enough so that in 10 minutes only about a maximum of 2 per cent error results from this cause (6). However, the possibility of enzymatic oxidation presents a possible deviation which was evaluated by setting up a series of experiments in which aspartate and α-ketoglutarate were eliminated from the reaction mixture, and different levels of oxalacetate were added. Aniline citrate was added at zero time in one flask and after 10 minutes incubation at 37.0° in a second flask (Table I). Evolutions of carbon dioxide were the same within experimental error in both flasks at several tissue levels, indicating that no signifi-

cant destruction of oxalacetate could be attributed to the tissue homogenate when manometric techniques were used.

The obvious type of control flask, in which α-ketoglutarate only is omitted, was found to be unsatisfactory since it did not yield a linear curve when tissue concentration was plotted against corrected evolution. A method of determining carbon dioxide evolution at zero tissue concentration was developed which is similar to that used by Schneider and Potter

TABLE I

Effect of Tissue Homogenate on Rate of Disappearance of Oxalacetic Acid

Sample No.	Liver (wet weight)	CO₂ evolution	
		Determination at zero time	Determination after 10 min. incubation at 37.0°
	mg.	*c.mm.*	*c.mm.*
1	20	205	208
2	50	207	217
3	100	227	219

The components of the reaction mixture are described in the text, except that the α-ketoglutarate and aspartate were omitted and 0.50 ml. of 0.020 M oxalacetate was initially present. A 10 per cent homogenate of rat liver was used.

TABLE II

Transaminase Activity of Representative Tissues from Normal Rats

Tissue	Q_T^{10} (oxalacetate appearance)	
Heart muscle............................	237	
	266	Average = 247
	251	
	234	σ = 12.7
Kidney cortex...........................	94.5	" = 4.9
Liver...................................	68	" = 4.0
Lung...................................	16	

(13) for the assay of cytochrome oxidase. It consists of plotting the carbon dioxide evolution, corrected for CO_2 retention in the tissue (previously determined), against the tissue concentration for three or more tissue levels and extrapolating to the axis to obtain the CO_2 evolution at zero tissue concentration (Fig. 1). This intercept value is subtracted from each of the experimentally obtained CO_2 evolutions to obtain the true CO_2 evolution as a function of the tissue level employed.

The calculation of the transaminase activity from the uncorrected carbon dioxide evolution obtained as described in a previous paragraph entails two

steps. First a correction is subtracted to account for the CO_2 retention in the tissue itself and amounts to 0.4 c.mm. of CO_2 per mg. of tissue on a wet weight basis. Secondly, these corrected CO_2 evolutions are plotted against tissue concentrations for three or more tissue levels. The CO_2 evolution at zero tissue level obtained by extrapolation is subtracted from each of the several corrected CO_2 evolutions to yield a value of CO_2 evolution which can be related directly to oxalacetate formed and therefore to enzyme present.

The transaminase level in the heart muscle of a series of white rats was determined to ascertain the experimental error of the determination. With four animals, an average Q_T^{10} was found to be 247 with a σ of 12.7. Table II shows the transaminase values obtained for a number of tissues from normal rats and, in cases in which the precision was determined, it was found to correspond with that given for heart muscle.

DISCUSSION

The proposed method has proved satisfactory for a number of different tissues of both plant and animal origin. In addition to normal rats the tissues from rats on a vitamin B_6-deficient diet have been analyzed in a satisfactory fashion. The technique has been used by Burris and Kreko[2] for the study of transaminase in the embryonic tissues from wheat, oats, and barley. It is anticipated that by ascertaining the possible errors, as indicated in the experimental section, this technique may offer a rapid and precise method of comparing the transaminase activity of tissues from both plants and animals.

Since this method is adapted for tissue preparations from the one proposed expressly for purified enzymes by Green et al. (6), the question of the method of expression of results is raised. The Q notation has become well established and accepted in enzyme literature. Cohen and Hekhuis (1) used the Q_T value exclusively, but this notation suffers when used for transaminase, since there is no indication of the incubation time and the Q_T varies markedly with the time of incubation. Green et al. (6) have standardized the incubation time and proposed as a unit of transaminase activity an amount of enzyme which produces "an amount of oxalacetic acid equivalent to 100 c.mm. of CO_2 formed per 10 minutes at 38°." However, when applied to tissues these units must be referred to the weight of tissue on a dry weight basis, and for this purpose it is not as convenient as the well standardized Q notation.

In order to obviate the difficulty experienced in relating the Q_T to the time of incubation, it is proposed that the time in minutes be indicated by a superscript. Thus Q_T^{10} would indicate the c.mm. of carbon dioxide liberated

[2] Burris, R. H., and Kreko, M. J., private communication.

from an equivalent amount of oxalacetate formed in a 10 minute incubation period per mg. of dry weight of tissue added. It is believed that this convention offers some measure of reproducibility in a system in which the rate of the reaction varies with incubation time.

SUMMARY

1. A rapid, precise, analytical method for the determination of aspartic-glutamic transaminase activity in tissue homogenates is described, based on the determination of oxalacetic acid by the use of aniline citrate.

2. A number of sources of possible deviation have been evaluated, methods of correction have been devised, and the precision of the method determined.

3. The transaminase activity is reported for a number of tissues of normal albino rats.

4. The use of the notation Q_T^{10} is proposed in which the superscript indicates the incubation time in minutes.

BIBLIOGRAPHY

1. Cohen, P. P., and Hekhuis, G. L., *J. Biol. Chem.*, **140**, 711 (1941).
2. Cohen, P. P., *Biochem J.*, **33**, 551 (1939).
3. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
4. Westerkamp, H., *Biochem. Z.*, **263**, 239 (1933).
5. Krebs, H. A., *Biochem. J.*, **32**, 108 (1938).
6. Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, **161**, 559 (1945).
7. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
8. Ostern, P., *Z. physiol. Chem.*, **218**, 160 (1933).
9. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
10. Schlenk, F., Fisher, A., and Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **61**, 183 (1946).
11. Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, in press.
12. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 15 (1945).
13. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).

THE CONJUGATED, NON-PROTEIN, AMINO ACIDS OF PLASMA

II. A STUDY OF DEPROTEINIZING TECHNIQUES

By HALVOR N. CHRISTENSEN AND ELEANOR L. LYNCH

(From The Mary Imogene Bassett Hospital, Cooperstown, New York)

Analyses reported recently (1) showed small and variable concentrations of bound α-amino acid nitrogen, released by acid hydrolysis, in tungstic acid filtrates, trichloroacetic acid filtrates, and ultrafiltrates of human plasma. The similarity of the quantities found in ultrafiltrates and tungstic acid filtrates in two cases suggested that the conjugates were diffusible through cellophane. In the present communication evidence is presented to show that the amino acid combinations of human plasma filtrates include non-diffusible conjugates, presumably proteins, and hence that deproteinization by these agents is inadequate for the determination of peptides. Serum, on the other hand, was found to contain not only more non-diffusible combined amino acids soluble in TCA (trichloroacetic acid) than the plasma from which it was derived, but also substantial quantities of amino acid combinations diffusible through cellophane.

Another question which concerned the validity of our analyses was studied first: Are the proteins of plasma stable under the conditions of sampling and deproteinization? The possibility that proteolysis occurred either before or during deproteinization must be considered, since (*a*) proteolytic proenzymes are present in plasma, which may be activated by chemical agents such as TCA (2–5); (*b*) increases in the conjugated amino acids soluble in TCA occur in the clotting of plasma (1).

The following criterion was proposed as evidence for freedom from interference by proteolysis. The conjugated amino acids found in filtrates should not be increased by reasonable prolongation in the periods of exposure to sampling or deproteinization conditions. This criterion was met with tungstic acid deproteinization. The total α-amino acid nitrogen values (by acid hydrolysis) were reproducible and constant for plasma for 3 to 5 hours after blood was drawn (Table I); the totals reached minimum values more than 10 minutes but less than 30 minutes after tungstic acid was added and showed no tendency to increase (Table II). 2.5 per cent TCA filtrates on the other hand reached minimum values so gradually (an interval of at least 2 to 8 hours between the addition of TCA and removal of the precipitate was required (Figs. 1 and 2)) that the possibility of proteolysis could not be excluded. The total α-amino nitrogen of TCA filtrates gradually approached the value for the tungstic acid filtrate of the

same plasma.   4 per cent TCA, a strength more frequently employed, re-
quired an hour or more to yield filtrates containing minimum total α-amino
nitrogen, with no evidence for a subsequent rise.   None of our plasma

TABLE I

*Effect of Varying Time Interval from Addition of Tungstic Acid to Removal of Protein
Precipitate by Centrifugation*

Values are in mg. per cent of α-amino N and each value is an average of two or
more analyses.

Plasma No.	Free α-amino nitrogen	Total α-amino nitrogen			
		Time interval			
		10 min.	30 min.	130 min.	180 min.
R77	4.51	5.14			4.91
R78	3.70	4.90			4.55
R84		4.52	4.37	4.40	4.39

TABLE II

*Reproducibility of Values Found for Total α-Amino Acid N in Duplicate Deprotein-
izations by Tungstic Acid; Effect of Storage of Plasma before Deproteinization*

The values are in mg. per cent of α-amino N and are averages of duplicates.   Each
total α-amino N represents a separate deproteinization.

Plasma No.	Interval from venipuncture to deproteinization, at room temperature				
	About 15 min.		2 hrs. Total	3 hrs. Total	5 hrs. Total
	Free	Total			
P88	3.91	4.40		4.48	
		4.39		4.43	
P90	3.74	4.25		4.26	
		4.24			
		4.25			
M4	4.29		4.67		4.67
M10	4.23	4.53			
		4.47			
M12	3.71	3.96			
		4.11			

filtrates became turbid upon standing.   In the experiments reported previ-
ously (1) the protein precipitate by TCA was filtered after 30 minutes
through a paper so small that 1 to 3 hours was required for filtration,
whereas in the present experiments the precipitates were removed by cen-
trifugation, followed by filtration of the supernatant.   Under the former
conditions the total α-amino nitrogen values were nearly minimum, whereas

if filtration was through a large paper and completed in a few minutes the values were much higher.
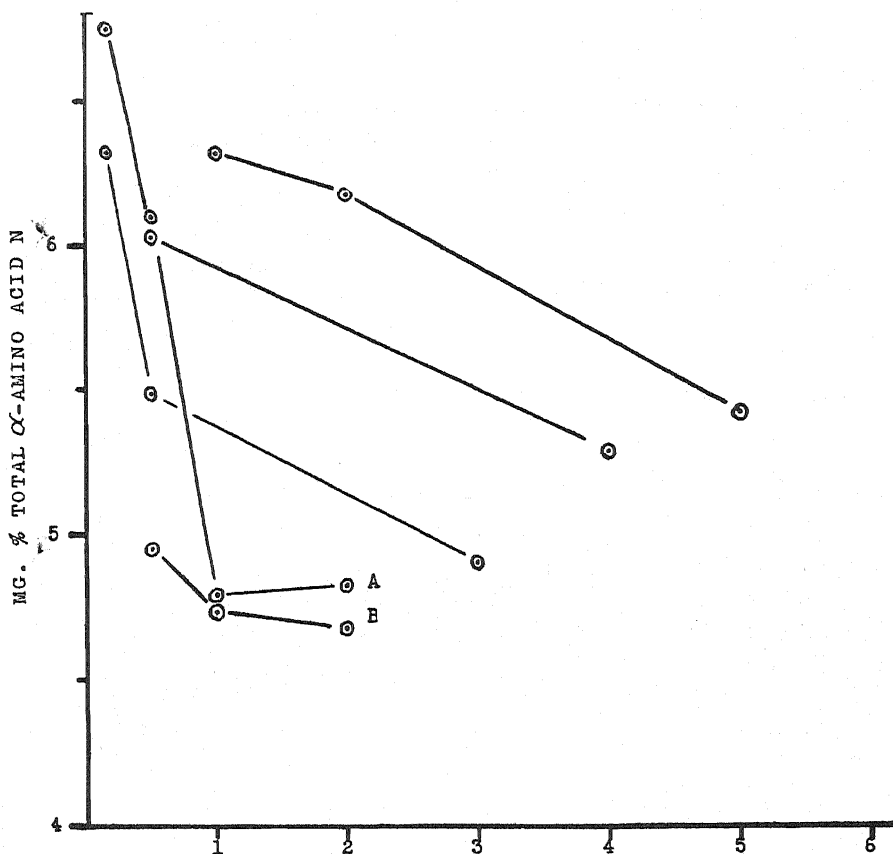
HOURS BEFORE PRECIPITATED PROTEIN REMOVED BY CENTRIFUGATION

FIG. 1. Decrease of total α-amino acid found with time of contact of plasma with trichloroacetic acid (TCA). Curve A, 4 per cent TCA by adding plasma to 9 volumes of TCA; Curve B, same plasma sample, 4 per cent TCA by adding 20 per cent TCA to diluted plasma. The other curves are for three different plasmas deproteinized by 2.5 per cent TCA.

The plasma-serum differences in conjugated α-amino nitrogen of TCA filtrates observed earlier (1) were uniformly observed in the present experiments, whether deproteinization by TCA proceeded for 30 minutes or 8 hours (Fig. 2; Tables III and IV). There was no evidence that heparin modified the rate of protein precipitation. These new soluble conjugates were observed within 15 minutes of drawing blood, before visible clotting

had occurred (Table III); hence their formation appeared to be an early event in blood coagulation. The conjugates were apparently not formed by fibrinolysis, since serum prepared by defibrination of plasma and immediately deproteinized contained as large a concentration as serum left in contact with the fibrin clot for 2 hours.

Do Deproteinizing Techniques Remove Proteins Completely?—This question was emphasized by the observation of Beckman, Hiller, Shedlovsky,
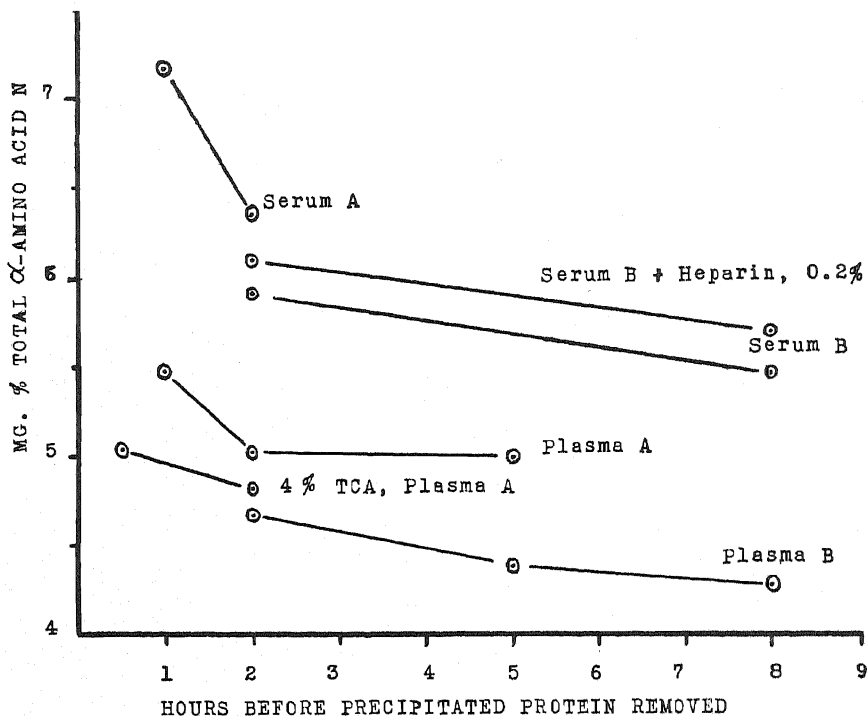
Fig. 2. Comparison of the behavior of plasma and serum with trichloroacetic acid. 2.5 per cent TCA except as indicated. Plasma B contained 3.76 mg. per cent of free α-amino N; Serum B (derived from Plasma B) contained 3.99 mg. per cent.

and Archibald (6) of a protein in urine soluble in 0.25 M TCA. We have found that within the error of measurement all of the conjugated α-amino nitrogen in tungstic or picric acid filtrates of six normal plasmas was not diffusible through cellophane (4 days with rotation) (Table IV). Picric acid filtrates of plasma were intermediate between tungstic acid filtrates and TCA filtrates in their content of conjugated α-amino nitrogen. The presence of dialyzable bound α-amino nitrogen in TCA filtrates was not as satisfactorily excluded as with the other two types of filtrates, but the concentrations were not over 0.04 to 0.3 mg. per cent.

TCA filtrates of serum on the other hand contained in three cases 0.70, 0.46, and 1.10 mg. per cent of diffusible conjugated α-amino nitrogen (Table IV). Of the total increase of conjugated α-amino nitrogen soluble in TCA produced by coagulation about half was diffusible.

Ultrafiltration and Dialysis of Plasma—In two former experiments (1) values for conjugated α-amino nitrogen in ultrafiltrates and tungstic acid filtrates were similar, suggesting that the latter reagent produced complete deproteinization. When, however, serial portions of ultrafiltrates were

TABLE III

Time Relationships in Formation of Extra, Trichloroacetic Acid-Soluble, Conjugated, α-Amino N in Coagulation of Plasma

Protein precipitates were removed 2 hours after adding trichloroacetic acid (TCA), except that with Plasma and Serum Q98, the 2.5 per cent TCA precipitate was removed after 5 hours. Sera Q98 and K38 were deproteinized 2 hours, Serum L34, $\frac{1}{2}$ hour, after coagulation began. Plasma K38 was defibrinated by whipping for a minute and the serum deproteinized at once.

Values are in mg. per cent of α-amino N and are averages of duplicates.

	Sample	Picric acid filtrate. Free α-amino N	Tungstic acid filtrate. Total	2.5 TCA filtrate. Total	4 per cent TCA filtrate. Total
Q98	Plasma, heparinized at once		4.17	5.21	4.85
	" " after 15 min.			6.22	
	Serum		4.96	6.24	5.74
L34	Plasma, heparinized at once	4.16		5.07	
	" " after 5 min.			5.09	
	" " " 15 "			5.15	
	Serum	4.20		5.99	
K38	Plasma	4.07		5.05	
	Serum by defibrination			5.84	
	" as usual	4.16		5.76	

analyzed, the filtrate formed during the first 6 hours was found to contain less total α-amino nitrogen (4.27 mg. per cent) and that formed during the subsequent 12 hours more (4.60 mg. per cent) than did a tungstic acid filtrate (4.42 mg. per cent). During the interval required to obtain adequate filtrate, proteolysis apparently occurred; so that the conjugated α-amino nitrogen found would vary with the length of time allowed for ultrafiltration. Dialysates of normal plasma prepared according to Hamilton and Archibald (7) (with 3 to 4 hours of dialysis) contained practically no conjugated α-amino nitrogen, although serum again showed diffusible amino acid conjugates (Table V), in this case without the intervention of chemical reagents other than water.

TABLE IV

Diffusibility through Cellophane of Conjugated α-Amino Nitrogen of Various Filtrates of Plasma and Serum; Comparison of Deproteinizing Agents

Values are in mg. per cent of α-amino N and are averages of duplicates.

| | Tungstic acid | | | Picric acid | | | 2.5 per cent trichloroacetic acid | | Interval in contact with trichloroacetic acid |
| | Free | Bound | | Free | Bound | | Bound | | |
		By differ-ence*	By dialy-sist		By differ-ence*	By dialy-sist	By differ-ence*	By dialy-sist	
									hrs.
Plasma Q95		0.75		3.67	0.75				
" M1		0.49		3.99	0.51				
" M4		0.37		4.29	0.34				
" M6		0.15	0.16	3.59					
" M10		0.24	0.19	4.23	0.32	0.28			
" M12		0.40	0.35	3.71	0.63				
" M14		0.26	0.42	3.30	0.30	0.31			
" M19‡				3.76			0.98	0.88	2
" N22	4.32	0.36	0.34		0.33		0.69		2
" M8	4.30	0.09					1.96	1.67	0.5
" M8							0.67	0.63	5
Serum M8	4.41	0.55					2.03	1.33	5
Plasma M16		0.23		4.02	0.38		1.05	0.89	5
Serum M16				4.23			1.92	1.46	5
" N20				4.19			1.93	0.83	2

* The increase in α-amino nitrogen produced by acid hydrolysis.

† The non-diffusible total α-amino N, by exhaustive dialysis of filtrates.

‡ Carcinoma of pancreas; serum bilirubin 19 mg. per cent; serum alkaline phosphatase 46 King units. Sample obtained through the courtesy of Dr. G. M. Mackenzie.

TABLE V

Dialyzable Conjugated α-Amino Acid N of Plasma and Serum

Direct dialysis in the apparatus of Hamilton and Archibald (7). Values are mg. per cent of α-amino N and are averages of duplicates.

Sample	Volume dialyzed	Period of dialysis	Free α-amino N, picric acid filtrate	Total α-amino N, dialysate	Conjugated diffusible α-amino N (by differ-ence)
	ml.	*hrs.*			
Plasma P90	3	4	3.74	3.74	0.00
Serum L34	3	3.5	4.20	4.81	0.61
Plasma K38	2	3	4.07	4.14	0.07
Serum K38	2	3	4.16	4.93	0.77
Plasma K35*	3	3	2.37	2.89	0.52

* From a patient, age 3 years, 22 hours after burns covering 28 per cent of the body surface. Sample obtained through the courtesy of Dr. M. A. McIver and Dr. R. D. Johnson.

EXPERIMENTAL

Plasma was separated from venous blood of fasting persons and collected in a heparin-containing syringe. For the preparation of serum, blood was centrifuged in a paraffined tube and the plasma separated and permitted to clot. After 2 hours the serum was separated. The heparin-containing plasma was also allowed to stand 2 hours before deproteinization when plasma and serum were being compared. Ten normal donors are represented in these experiments.

Procedures employed previously (1) were modified as follows: Protein precipitates were separated by centrifugation, 15 and 30 minutes after addition of the precipitant in the case of picric and tungstic acids, respectively, unless otherwise indicated. The supernatant solutions were then filtered. Hydrolyses were performed in sealed tubes in an oven for 24 hours at 105°. Corrections for the CO_2 evolution from urea were calculated on the basis of urea estimations (8). At the suggestion of Dr. Hamilton, hydrazine sulfate was included in the 2 N lactic acid solution rather than in 0.5 N sodium hydroxide, in the manometric estimation of α-amino nitrogen.

Picric acid was used for deproteinization according to the directions of Hamilton and Van Slyke (8). The removal of picric acid before acid hydrolysis proved necessary. Aliquots of filtrates were made to 0.2 N in hydrochloric acid and extracted in a continuous extractor for 4 hours with peroxide-free ether (this was twice the interval required to eliminate the color of picric acid). The aqueous solutions were concentrated *in vacuo* to remove ether before hydrolysis. Glutamine added to plasma was recovered satisfactorily as total α-amino nitrogen by our procedures with all three protein precipitants.

DISCUSSION

According to our interpretation the results described here vitiate all evidence advanced heretofore for the normal or pathological presence of polypeptides in plasma. In those investigations in which reliance has not been placed upon chemical deproteinizing agents, serum was employed and dialysis periods were apparently prolonged (9, 10). We are investigating whether or not dialyzable, conjugated amino acids are present in body fluids in disease, and how long they persist in plasma after the intravenous administration of partially hydrolyzed proteins.

The amino acid conjugates of plasma which are soluble in tungstic and picric acids have some of the properties of proteins and may be related to the globulin-like material soluble in trichloroacetic acid, observed in the urine of a number of patients (6). Proteins soluble in TCA were said to be present also in plasma. With tungstic acid deproteinization at least,

the amount of conjugated amino nitrogen found was reproducible but showed a considerable range of variation. Tungstic acid filtrates have shown no turbidity upon being brought to 0.5 per cent with picric acid or to 0.25 M with TCA, suggesting that the fraction present in tungstic acid filtrates was also present in the other two types of filtrates.

An early event in the clotting of plasma appears to be the formation of amino acid conjugates soluble in TCA, part diffusible through cellophane, part not diffusible. Both of these moities may be products of proteolytic action upon plasma proteins. Interestingly, the diffusible compounds were precipitated in deproteinization by tungstic acid. The prevention of this event does not require the addition to plasma of an antiproteolytic agent (which heparin appears to be (11, 5)) since oxalate also prevents the change (1).

Analytical Implications—The foregoing observations have several implications for the precise determination of non-protein nitrogen in biological material too obvious to require discussion.

The following procedure is proposed for the estimation of conjugated non-protein α-amino acids in fluids or tissues. The material is immediately extracted with picric or trichloroacetic acid (at least in the case of serum (1) and of peptone (12), the latter is more inclusive). The filtrates are then acidified and extracted with ether to remove the deproteinizing agent, concentrated *in vacuo* to remove ether, and aliquots dialyzed against a measured volume of water. Aliquots of the dialysate are hydrolyzed by acid and the *total* diffusible α-amino nitrogen determined. The *free* α-amino nitrogen is determined promptly upon the original picric acid filtrate, or upon the ether-extracted TCA filtrate. This procedure attempts to eliminate proteolytic action by removing most of the protein before dialysis is undertaken.[1] For the analysis of plasma, blood should be drawn with a heparin-containing syringe. Dialysates prepared according to Hamilton and Archibald (7) of plasma and other fluids are suitable for examination for conjugated amino acids, except that, in case of a positive finding, proteolytic action during dialysis must be ruled out. Thus it is not known whether the plasma of the burned patient of Table V contained peptides or increased proteolytic activity.

SUMMARY

1. Filtrates of plasma by tungstic acid contain reproducible amounts of conjugated α-amino acids. The amounts were not varied by storing the

[1] Further application of these procedures has indicated that the plasma of normal persons contains dialyzable amino acid conjugates at some times and not at others. Non-dialyzable conjugates probably cannot account for all of the high concentrations of conjugates observed in some tungstic acid filtrates in our earlier communication (1).

plasma 3 to 5 hours or by several hours contact with the precipitant. This constancy is taken as evidence that proteolysis during handling does not contribute appreciably to the total α-amino nitrogen found.

2. Plasma filtrates by tungstic, picric, and trichloroacetic acids contained increasing amounts of conjugated amino acids in the order named.

3. Filtrates of plasma by tungstic, trichloroacetic, and picric acids contained appreciable amounts of conjugates not diffusible through cellophane. These substances accounted, in several cases, for all of the conjugated amino acids of plasma. Hence deproteinization by these agents cannot be relied upon for the determination of plasma peptides.

4. The conjugated α-amino nitrogen of trichloroacetic acid filtrates of plasma decreased with increase of the interval between addition of the precipitant and removal of the precipitate, for 2 to 8 hours with 2.5 per cent trichloroacetic acid and for an hour with 4 per cent.

5. The clotting of plasma produced increases in the conjugated α-amino acids of trichloroacetic acid filtrates. The increase was observed in some cases before coagulation was evident, and was not dependent upon contact between serum and fibrin. The increases were not significantly modified by varying the time interval for deproteinization or the strength of the trichloroacetic acid. Of the extra conjugated α-amino nitrogen in the filtrates about half was diffusible through cellophane. Brief dialysis of serum itself also demonstrated diffusible amino acid combinations. Thus serum, in contrast to plasma, contained considerable concentrations of conjugated, dialyzable, α-amino acids.

BIBLIOGRAPHY

1. Christensen, H. N., and Lynch, E. L., J. Biol. Chem., 163, 741 (1946).
2. Hedin, S. G., Z. physiol. Chem., 104, 11 (1919).
3. Schmitz, A., Z. physiol. Chem., 250, 37 (1937).
4. Tagnon, H. J., J. Lab. and Clin. Med., 27, 1119 (1942).
5. Rocha e Silva, M., and Andrade, S. O., Science, 102, 670 (1945).
6. Beckman, W. W., Hiller, A., Shedlovsky, T., and Archibald, R. M., J. Biol. Chem., 148, 247 (1943).
7. Hamilton, P. B., and Archibald, R. M., Ind. and Eng. Chem., Anal. Ed., 16, 136 (1944).
8. Hamilton, P. B., and Van Slyke, D. D., J. Biol. Chem., 150, 231 (1943).
9. Kalmykoff, M. P., Arch. ges. Physiol., 205, 493 (1924).
10. Abderhalden, E., and Rossner, E., Fermentforschung, 10, 102 (1928).
11. Horwitt, M. K., Science, 92, 89 (1940).
12. Hiller, A., and Van Slyke, D. D., J. Biol. Chem., 53, 253 (1922).

THE INACTIVATION OF DIETHYLSTILBESTROL IN VITRO

By HELEN ZIMMERBERG

(*From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*)

Heller (1) has reported that estrone and estradiol are inactivated by liver slices and liver mince. Zondek *et al.* (2) confirmed this result and also demonstrated that estrone is inactivated more rapidly than stilbestrol. In his work with estradiol Heller has shown that oxidation, and not conjugation, is responsible for the loss of biological activity. Bernheim and Bernheim (3) have observed that phenol is inactivated by guinea pig liver slices and that both conjugation and oxidation are responsible. Using liver slices from one strain of rats, DeMeio and Arnolt (4) found that conjugation of phenol could take place with either sulfuric or glucuronic acid. The results obtained by these workers suggested the possibility that stilbestrol might be oxidized or conjugated, and therefore its inactivation by rat liver slices *in vitro* was studied in detail.

EXPERIMENTAL

Rat liver slices were used in most of the experiments. They were suspended in Krebs' bicarbonate solution which was saturated with a 95 per cent O_2-5 per cent CO_2 gas mixture. The experiments were carried out in this atmosphere with the slices suspended in 4.0 cc. of solution in 50 cc. Erlenmeyer flasks and shaken at 37°. 10 mg. of diethylstilbestrol were rubbed into a paste with 0.15 cc. of 10 per cent NaOH. 2 cc. of water were then added and the suspension stirred until all of the material was in solution. The solution was made up to 9 cc., 4 N HCl were added until a faint cloudiness resulted, and finally it was made up to 10 cc.

Stilbestrol was added to the flasks just before they were put in the bath. Experiments were set up in duplicate with controls to which no stilbestrol was added and controls to which stilbestrol was added at the end of the incubation period. 1 cc. of 20 per cent trichloroacetic acid was added to all the vessels and allowed to act for 10 minutes, and then 4 cc. of 95 per cent ethyl alcohol were added. Without the alcohol it is impossible to recover the stilbestrol, for it is readily adsorbed on the slices. After 10 minutes in alcohol the solution was decanted, centrifuged, and the stilbestrol remaining in the liquid estimated by the Tubis and Bloom (5) modification of the Folin and Ciocalteu method (6). When stilbestrol was added at the end of the incubation period to unboiled tissue, 98 to 100

per cent was recovered. The same recovery was obtained when stilbestrol was incubated with boiled tissue. The accuracy of the estimation is within 5 per cent.

In Tables I to III the "Amount added" represents the amount recovered from those vessels to which the stilbestrol was added at the end of the incubation period. This eliminates errors in decanting. For the hydrolysis the centrifugate was divided into thirds. One part was analyzed as such. To another 0.3 cc. of concentrated HCl was added and the solution autoclaved for 15 minutes at 24 pounds pressure. To the third part 0.3 cc. of concentrated HCl was added and the solution placed in a boiling water bath for 30 minutes. Both portions were neutralized with NaOH and the solutions transferred to 100 cc. volumetric flasks with two washings of 1 cc. 95 per cent ethyl alcohol each. The stilbestrol was then estimated in each of the three parts.

There is some loss of free stilbestrol on autoclaving and boiling, and to take this factor into account the centrifugate from the vessels to which stilbestrol was added after the incubation period was always autoclaved (or boiled) at the same time and under the same conditions as the experimental tubes. When the solution is autoclaved for 1 hour instead of 15 minutes, the percentage conjugation remains the same. Therefore, the assumption seems justified that the conjugated stilbestrol is rapidly hydrolyzed and that the loss in the experimental tubes is the same as in the control. The amount of stilbestrol left in these control tubes is considered the amount added for those parts of the experiment in which these techniques are used.

Under the conditions of the experiments, half of the stilbestrol disappears rapidly when added to liver slices, but never more than this. This value is constant when 0.05 to 0.4 mg. of stilbestrol is incubated with 300 to 600 mg. of tissue slices and reaches its maximum during the first half-hour. With 150 mg. of tissue, the maximum is reached in 3 hours. These results are shown in Table I and Fig. 1.

The color developed in the estimation of stilbestrol represents the action of both the hydroxyl groups, since both are equally capable of reducing the reagent. Therefore, it seems likely that the apparent disappearance of no more than 50 per cent of the added stilbestrol means that, although all the molecules react, only one of the hydroxyl groups per molecule takes part in the reaction.

The disappearance of the stilbestrol might be the result of oxidation or conjugation. If conjugation occurs, the stilbestrol should be recovered after autoclaving, and this happens when the concentration of stilbestrol is small (up to 0.2 mg. per 4.0 cc.; see Table II). With larger amounts, the proportion which is recoverable decreases, although the percentage dis-

TABLE I

Effect of Various Conditions on Disappearance of Stilbestrol

Tissue slices	Time of incubation	Amount added	Amount recovered	Disappeared
mg.	*hrs.*	*mg.*	*mg.*	*per cent*
150	0.5	0.385	0.296	22
150	3.0	0.100	0.050	50
150	3.0	0.390	0.192	51
300	0.5	0.109	0.053	50
300	0.5	0.368	0.193	48
300	3.0	0.049	0.023	53
300	3.0	0.197	0.100	49
300	3.0	0.410	0.223	46
600	3.0	0.138	0.070	49
600	3.0	0.394	0.200	49
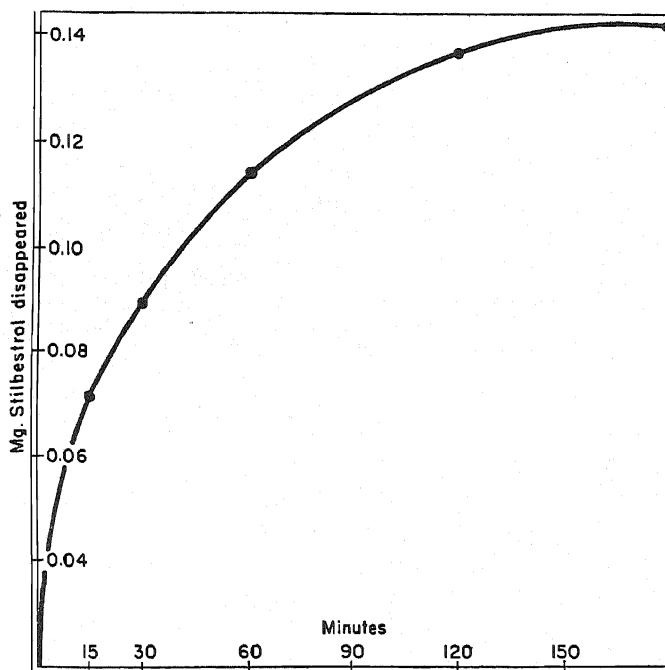
FIG. 1. The disappearance of 0.39 mg. of stilbestrol incubated with 160 mg. of rat liver slices at 37°. The tissue controls have been subtracted.

appearance does not change. This suggests that there is a limit to the amount of stilbestrol which can be conjugated, and that another mechanism, probably oxidation, is available under these conditions.

It has been shown that phenol can be conjugated with sulfuric or glucuronic acids. The sulfate esters are readily hydrolyzed by boiling, but the glucuronates require autoclaving. Under the conditions described above, about half of the stilbestrol which disappears can be recovered on boiling (see Table II) and the other half after autoclaving. If, however, the liver slices are suspended in Ringer's solution in which the $MgSO_4$ is replaced by $MgCl_2$, the amount which can be hydrolyzed by boiling is very much smaller. This small amount probably represents the use of sulfate already

TABLE II

Effect of Concentration of Stilbestrol on Percentage Conjugated by 160 Mg. (Wet Weight)
of Rat Liver Slices Incubated 3 Hours at 37°

The contents of each vessel were divided into three equal parts. Therefore, Column 1 represents one-third of the total amount added and Columns 4 and 7 show the loss due to autoclaving and boiling respectively. The tissue controls have been subtracted.

Amount added	Amount recovered	Disappeared* (control)	Amount added (auto-claved)	Amount recovered after autoclaving	Conjugated†	Amount added (boiled)	Amount recovered after boiling	Conjugated†
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
0.077	0.044	43	0.071	0.068	91	0.071	0.053	42
0.094	0.058	39	0.083	0.075	69	0.078	0.058	33
0.120	0.073	40	0.105	0.085	52	0.112	0.081	30
0.140	0.081	41	0.126	0.095	39	0.121	0.081	15

* Per cent disappeared = 100 − (amount recovered)/(amount added) × 100.

$$\dagger \ Per \ cent \ conjugated = \frac{(\% \ disappeared \ in \ control) - (\% \ disappeared \ on \ autoclaving)}{\% \ disappeared \ in \ control}$$

present in the slices (see Table III). At the same time the amount hydrolyzed by autoclaving is decreased, although conjugation is still taking place to a considerable extent. Since part of the conjugation requires the presence of sulfate, it can be concluded that a sulfate ester is formed. The part that does not require sulfate and which requires autoclaving for the recovery of the free stilbestrol may be a glucuronate ester.

The relationship of the weight of slices used in each vessel to the conjugation was studied and it was found that with 0.1 mg. of stilbestrol the per cent conjugated was the same for 150, 300, and 600 mg. of tissue slices. This was also true when 0.4 mg. was used. However, as shown by Table I, the time required for completion of the reaction is decreased as the weight of the tissue slices increases.

Kidney slices cause little or no disappearance of stilbestrol. When there

is some disappearance there is no evidence of conjugation. 98 per cent of the stilbestrol incubated with boiled liver slices or with cyanide is recovered. The results indicate that the mechanisms responsible for the disappearance are thermolabile and are inhibited by cyanide.

Liver suspensions were studied in order to determine whether conjugation would occur in broken cells. It has been reported (2) that liver suspensions inactivate 80 per cent of the added stilbestrol, estimated by its biological activity. To prepare the suspensions 6 gm. of rat liver were cut

TABLE III

Effect of Presence Or Absence of Sulfate Ion on Amount of Stilbestrol Conjugated

0.25 mg. of stilbestrol was incubated with 160 mg. of rat liver slices for 3 hours at 37°. The contents of each vessel were divided into three equal parts. Therefore Column 1 represents one-third of the total amount added and Columns 4 and 7 show the loss due to autoclaving and boiling respectively. The tissue controls have been subtracted.

Amount added	Amount recovered	Disappeared*	Amount added (autoclaved)	Amount recovered after autoclaving	Conjugated†	Amount added (boiled)	Amount recovered after boiling	Conjugated†	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	
0.083	0.054	35	0.071	0.054	34	0.074	0.048	7	No sulfate
0.082	0.048	42	0.068	0.051	36	0.070	0.046	14	
0.083	0.057	31	0.070	0.056	35	0.072	0.050	0	
0.077	0.044	43	0.071	0.068	91	0.071	0.053	42	Sulfate
0.081	0.053	35	0.082‡	0.084	100	0.080‡	0.062	37	

* See foot-note to Table II.
† See foot-note to Table II.
‡ In this particular sample there was no destruction of the stilbestrol after autoclaving or boiling. In all other experiments the destruction of stilbestrol with or without sulfate varied between 10 and 15 per cent.

into small pieces, added to 35 cc. of Ringer-bicarbonate solution previously saturated with 95 per cent O_2-5 per cent CO_2 gas mixture, and ground in a Waring blendor for 30 seconds. 4 cc. of the resulting suspension were used in each vessel and the appropriate amount of stilbestrol added to the experimental vessels. The same types of controls were set up for the suspension as for slices. The results can be summarized as follows: After 3 hours incubation with 0.3 mg. of stilbestrol 32 to 48 per cent had disappeared and after autoclaving none could be recovered. Therefore, intact cells are necessary for conjugation, although oxidation can occur in broken cells.

DISCUSSION

Heller has observed no conjugation of estradiol by liver slices or liver mince. In the present experiments it has been shown that conjugation is the preferred method of inactivating stilbestrol. It apparently is oxidized only when the liver cannot handle it by conjugation. With a strain of rats found by Bernheim and Bernheim to give no conjugation of phenol with glucuronic acid, these experiments have shown that stilbestrol is conjugated by sulfuric and probably also glucuronic acid, and that these two are utilized equally.

Heller found that cyanide was the only poison of the large number he studied which inhibited the inactivation of estradiol. This observation on the action of cyanide has been confirmed in these experiments with stilbestrol. The absence of conjugation as well as oxidation in the presence of cyanide indicates the need of a coupled reaction that will provide the energy for the formation of the conjugate. This may be an explanation for the inability of broken cells to conjugate stilbestrol.

SUMMARY

1. Stilbestrol disappears when added to rat liver slices in Ringer-bicarbonate solution shaken at 37° in 95 per cent CO_2-5 per cent O_2.

2. The stilbestrol that disappears is either conjugated or oxidized, the amount of each depending upon the concentration of stilbestrol. Apparently only one of the two hydroxyl groups is attacked.

3. Thermolabile enzyme systems sensitive to cyanide are responsible for the conjugation and oxidation of stilbestrol.

4. The conjugation can take place with sulfuric or another, probably glucuronic, acid, depending upon which is available.

5. Intact cells are necessary for conjugation but not for oxidation.

BIBLIOGRAPHY

1. Heller, C. G., *Endocrinology*, **26**, 619 (1940).
2. Zondek, B., Sulman, F., and Sklow, J., *Endocrinology*, **33**, 333 (1943).
3. Bernheim, F., and Bernheim, M. L. C., *J. Pharmacol. and Exp. Therap.*, **78**, 394 (1943).
4. DeMeio, R. H., and Arnolt, R. I., *J. Biol. Chem.*, **156**, 577 (1944).
5. Tubis, M., and Bloom, A., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 309 (1942).
6. Folin, O., and Ciocalteu, J., *J. Biol. Chem.*, **73**, 627 (1927).

THE UTILIZATION OF THE OPTICAL ISOMERS OF PHENYL-ALANINE, AND THE PHENYLALANINE REQUIREMENT FOR GROWTH*

By WILLIAM C. ROSE AND MADELYN WOMACK

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

In the use of diets containing mixtures of amino acids in place of proteins one is frequently confronted with the necessity of employing synthetic amino acids, since the latter may be more readily available than the optically active forms which occur in proteins. Therefore, it becomes of considerable practical importance, as well as of theoretical interest, to determine the relative availability in the animal organism of the enantiomorphs of the essential amino acids.

Du Vigneaud *et al.* (9) and Berg (1) have shown that the optical isomers of tryptophane possess equal nutritive value for the rat. This is true also of histidine, except that the dextrorotatory form appears to be slightly less effective than the naturally occurring $l(-)$ type (Cox and Berg (2)). Jackson and Block (4) have reported that both $l(-)$- and $d(+)$-methionine successfully supplement diets low in methionine and cystine. This conclusion has been confirmed in unpublished experiments in this laboratory under conditions which preclude the presence of more than minute traces of either cystine or methionine in the basal ration.

The present paper[1] is concerned with a comparison of the growth effects of $l(-)$- and $d(+)$-phenylalanine, and with a semiquantitative estimate of the minimum amount of this amino acid which is capable of inducing optimum increases in weight.

EXPERIMENTAL

Weanling rats served as the experimental subjects throughout. These were housed in individual cages and were permitted to consume the food *ad libitum*. All amino acids were purified until they yielded correct analytical values.

In the first experiments the nitrogen of the basal diet was derived from a

mixture of amino acids (Mixture XVI-b, Table I) which was devoid of phenylalanine, tyrosine, and threonine. The natural isomer of the latter was added to the diet separately. As will be observed from Table I, when racemic acids were used in Mixture XVI-b the quantities were doubled in order to allow for the unnatural enantiomorphs. The basal diet (Diet 1,

TABLE I

Composition of Amino Acid Mixtures

	Mixture XVI-b		Mixture XX-b	
	Physiologically active	As used	Physiologically active	As used
	gm.	*gm.*	*gm.*	*gm.*
Glycine........................	0.50	0.50	0.1	0.1
Alanine.......................	1.90	3.80*	0.2	0.4*
Serine........................	0.50	1.00*	0.1	0.2*
Valine........................	8.00	16.00*	1.0	2.0*
Leucine.......................	9.00	18.00*	1.3	2.6*
Isoleucine.....................	4.00	8.00*	0.9	1.8*
Cystine.......................	1.25	1.25	0.2	0.2
Methionine....................	1.75	3.50*	0.6	0.6*
Threonine.....................	0	0	0.7	1.4*
Proline.......................	2.00	2.00	0.2	0.2
Hydroxyproline................	0.30	0.30	0.1	0.1
Tryptophane...................	2.25	2.25	0.4	0.4
Aspartic acid..................	4.10	4.10	0.2	0.2
Glutamic "	22.00	22.00	2.0	2.0
Lysine........................	7.70		1.5	
" monohydrochloride.....		19.25*		1.9
Histidine......................	3.70		0.5	
" monohydrochloride monohydrate................		5.00		0.7
Arginine.......................	5.25		0.5	
" monohydrochloride		6.35		0.6
Sodium bicarbonate............		13.38		1.3
	74.20	126.68†	10.5	16.7

* Racemic acids.

† 1.707 gm. of Mixture XVI-b contained 1.0 gm. of natural amino acids.

Table II) contained 18 per cent of natural amino acids, including threonine. When one of the antipodes of phenylalanine was included in the ration, the quantity of active amino acids was maintained at 18 per cent by an appropriate reduction in the amount of Mixture XVI-b. With this diet, the vitamin B factors were supplied by two pills which were fed separately to each animal daily. Each pill contained 75 mg. of a commercial vitamin concentrate (*cf.* Table II).

The optical isomers of phenylalanine were prepared by resolution of *dl*-phenylalanine through the brucine salt of the formyl derivative according to the du Vigneaud and Meyer (8) modification of the Fischer and Schoeller (3) procedure.   The $d(+)$-phenylalanine thus prepared showed a rotation in aqueous solution of $[\alpha]_D^{24} = +34.8°$.   The values reported by du Vigneaud and Meyer (8) and Fischer and Schoeller (3) are $+33.5°$ and $+35.1°$

TABLE II

*Composition of Basal Diets**

	Diet 1	Diet 2
	gm.	*gm.*
Amino acid Mixture XVI-b................	29.5	
" " " XX-b................		16.7
Threonine†................................	0.7	
Dextrin....................................	17.8	31.3
Sucrose....................................	15.0	15.0
Salt mixture‡..............................	4.0	4.0
Agar.......................................	2.0	2.0
Lard.......................................	26.0	26.0
Cod liver oil..............................	5.0	5.0
	100.0	100.0

* Diet 1 contained 18 per cent of natural amino acids and Diet 2 10.5 per cent of physiologically active amino acids.   In the experiments involving the use of Diet 1, the vitamin B factors were furnished in the form of two pills daily, each containing 75 mg. of Vitab Products "Vitamin B complex liquid, type II."   In the experiments in which Diet 2 was used, the water-soluble vitamins were supplied in two pills daily, each containing either 75 mg. of milk concentrate and 50 mg. of tikitiki extract, or 10 γ of thiamine hydrochloride, 20 γ of riboflavin, 10 γ of nicotinic acid, 10 γ of pyridoxine hydrochloride, 50 γ of calcium d-pantothenate, 5 mg. of choline chloride, 1.5 mg. of p-aminobenzoic acid, 5 mg. of inositol, 25 mg. of wheat germ oil, and 12.5 mg. of Wilson's "liver powder 1:20."   No differences were observed in the growth effects of the two types of vitamin supplements.

† Natural threonine from blood fibrin.

‡ Osborne and Mendel (5).

respectively.   The $l(-)$-phenylalanine had a rotation in water of $[\alpha]_D^{26} = -34.4°$, as compared to a value of $-35.1°$ given by Fischer and Schoeller (3).   Both optical isomers yielded correct data on analysis.

The effects upon growth brought about by the supplementation of Diet 1 with $l(-)$- and $d(+)$-phenylalanine were tested in two litters of rats, and the results are summarized in Table III.   The growth curves of one litter are shown in Chart I.   When these experiments were undertaken, no information was available as to the percentage of dietary phenylalanine which is necessary to induce satisfactory growth.   For the first 8 days, 0.5 per

cent of each isomer was used. This proved to be inadequate, although the losses in weight were less severe than in the controls (Rats 2623 and 2629, Table III) which were deprived of phenylalanine. Thereafter, the level of phenylalanine was raised to 1.0 per cent, and growth ensued immediately. It will be observed that the subjects which received the $d(+)$-phenylalanine grew quite satisfactorily, although the total gains were not quite so large as

TABLE III

Comparative Growth Effects of $l(-)$- and $d(+)$-Phenylalanine

Litter No.	Rat No. and sex	Days	Change in body weight	Food intake	Supplements added to Diet 1
			gm.	*gm.*	
1	2619 ♂	8	−3	24	0.5% $l(-)$-phenylalanine
		28	+42	141	1.0% "
	2620 ♀	8	−2	23	0.5% "
		28	+38	154*	1.0% "
	2621 ♂	8	−3	26	0.5% $d(+)$-phenylalanine
		28	+31	143	1.0% "
	2623 ♀	8	−11	15	None
		28	−9	61	"
2	2624 ♂	8	+1	31	0.5% $l(-)$-phenylalanine
		28	+43	158	1.0% "
	2625 ♀	8	−2	27	0.5% "
		28	+38	158*	1.0% "
	2626 ♂	8	−2	20	0.5% $d(+)$-phenylalanine
		28	+35	123	1.0% "
	2627 ♀	8	−2	25	0.5% "
		28	+34	121	1.0% "
	2628 ♀	8	−2	24	0.5% "
		28	+29	117	1.0% "
	2629 ♀	8	−11	15	None
		28	−7	64	"

* The animal scattered food; the recorded intake is probably too high.

in the animals which received the $l(-)$-phenylalanine. Whether the differences are significant is not clear. In any event, a large proportion of the $d(+)$-phenylalanine was utilized for growth purposes, and must have undergone inversion in the organism.

In order to obtain further information concerning the relative effectiveness of the two isomeric modifications of phenylalanine, a second series of experiments was undertaken in which dl- and $l(-)$-phenylalanine were administered at several levels. In the meantime, amino acid Mixture XX-b had been developed in this laboratory and used successfully in a large number of tests. In this mixture (Table I) the proportion of dl-methionine
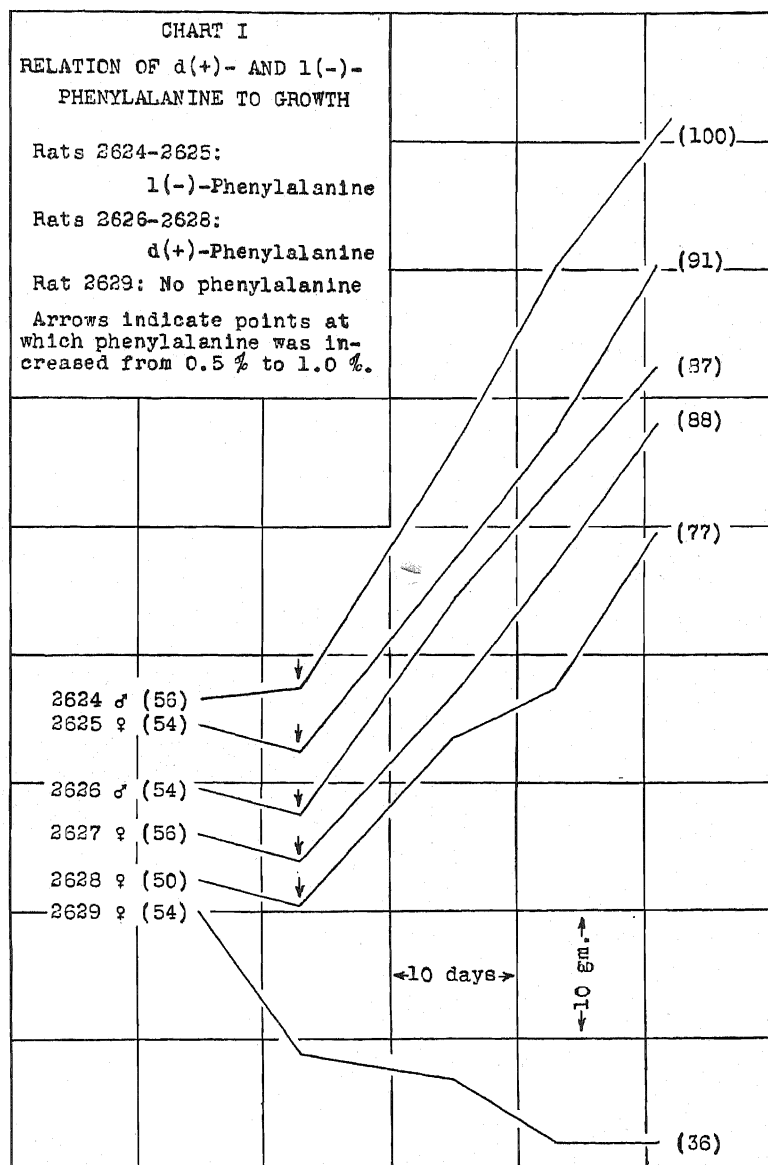
CHART I. The numbers in parentheses denote the initial and final weights of the rats.

was not doubled over the desired amount of the natural isomer since, as indicated above, both forms are readily utilized by the rat. Invariably, the diets in the second series of tests contained 16.7 per cent of Mixture

XX-b.   This furnished 10.5 per cent of physiologically active amino acids. The make-up of the basal ration (Diet 2) is shown in Table II.   When dl- or $l(-)$-phenylalanine was included in the food it replaced an equal weight of dextrin.   The slight variation in nitrogen content of the diets thereby resulting was without influence upon the growth rates.   Two types of vitamin supplements (*cf.* Table II) were employed in the experiments involving the use of Diet 2, but no differences in growth attributable to this variable could be detected.

The plan was to vary the percentage of dl- and $l(-)$-phenylalanine in the diet, and thereby determine approximately the minimum level of each which would induce maximum growth.   Obviously, if both optical isomers are equally available in the organism the amount of dl-phenylalanine found

TABLE IV

Comparative Growth Effects of dl- and $l(-)$-Phenylalanine at Different Dietary Levels

The experiments covered 28 days each.   The figures in parentheses represent the number of animals.

	Dietary phenylalanine, *per cent*.....	0.5	0.6	0.7	0.8	0.9	1.0	1.2
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
dl-Phenyl-alanine	Males		(5)	(8)	(12)	(4)	(9)	(2)
	Average gain		24.0	40.1	49.2	55.0	54.8	41.5
	Females		(4)	(9)	(13)	(4)	(4)	(1)
	Average gain		21.3	34.2	38.0	54.8	47.8	39.0
	Both sexes		(9)	(17)	(25)	(8)	(13)	(3)
	Average gain		23.0	37.0	43.4	54.9	52.6	40.7
$l(-)$-Phenyl-alanine	Both sexes	(3)	(6)	(12)	(9)	(7)		
	Average gain	20.7	29.3	37.3	45.1	53.1		

necessary for maximum increases in weight should not exceed the requirement of $l(-)$-phenylalanine.   Since the experiments with $l(-)$-phenylalanine described above showed that 0.5 per cent was insufficient and that 1.0 per cent permitted satisfactory growth, the levels used in the present tests were kept, for the most part, within these limits.   In each test the period of growth was 28 days.

Experiments of this nature were made on 112 animals.   Of these, 75 received dl-phenylalanine and thirty-seven $l(-)$-phenylalanine.   In order to conserve space, the average total gains only are presented.   These are summarized in Table IV.   With dl-phenylalanine the results are shown for each sex separately and for both sexes.   With $l(-)$-phenylalanine the smaller number of animals does not warrant a division according to sex, although to make such a division would not alter the findings.

An inspection of the data reveals the fact that the minimum level of *dl*-phenylalanine capable of inducing maximum growth is 0.9 per cent. This is true whether one considers the males only, the females only, or both sexes. Higher percentages are certainly no better, and actually appear to exert some inhibition in growth. However, with respect to the latter possibility, one should not overlook the fact that the number of animals on the highest level (1.2 per cent) was very small.

In the tests with *l*(−)-phenylalanine, each increment up to 0.9 per cent led to an improvement in growth. Unfortunately, higher percentages of this modification were not tested; but probably one is warranted in assuming that 0.9 per cent is the optimum level, for to conclude otherwise would imply that the natural isomer is *less* efficient than the racemic compound. The only obvious factor which might invalidate the latter concept is the rather remote possibility that *d*(+)-phenylalanine, in the concentrations employed, is toxic, and that the effects observed with *dl*-phenylalanine represent summations of the growth-stimulating and growth-inhibiting activities of the two optical isomers. We expect to look into this possibility at a later date. In the meantime, for practical feeding purposes and within the limits of accuracy of the methods employed, it appears that *dl*-phenylalanine (and therefore *d*(+)-phenylalanine) is almost, if not quite, as well utilized by the rat for growth purposes as is *l*(−)-phenylalanine. This is fortunate, since the latter is one of the more difficult amino acids to isolate in pure form from proteins.

A by-product of this investigation is the establishment of an approximate minimum level of dietary phenylalanine for the growing rat. Several years ago a table summarizing the evidence then available concerning the minimum levels of all of the indispensable amino acids was published by the senior author (Rose (6, 7)). At that time, emphasis was placed on the "strictly tentative" nature of the values, and the reader was reminded that "factors such as the proportion of fat and carbohydrate in the ration, and the age, weight and sex of the subjects may play important rôles in determining the minimum level of a given component." Despite these precautions, the data have been misinterpreted and incorrectly applied on more than one occasion. For this reason, we wish to emphasize anew that at best such minima are *approximations* only, albeit useful for many experimental purposes. It should be unnecessary to state that minima are applicable only to the species in which they are determined, in this case the rat. Their tentative nature is illustrated by the fact that originally (Rose (6)) the minimum level of phenylalanine was placed at 0.7 per cent of the diet. This was based on tests in a relatively small number of animals. The value is now revised upward to 0.9 per cent. Revisions affecting other amino acids may become necessary. Finally, minima ordinarily have been estab-

lished "when liberal quantities of the non-essential amino acids were furnished." This is necessary in order to avoid the "use of the indispensable amino acids for the manufacture of the dispensable group." In the case of methionine, the tests were conducted with a diet devoid of cystine, since approximately one-sixth of the methionine requirement for growth can be met by cystine (*cf.* Womack and Rose (10)). In the present study the diets were devoid of tyrosine. In a subsequent paper we shall show the extent to which the presence of tyrosine in the food reduces the phenylalanine requirement.

SUMMARY

Feeding experiments in which the basal diet was devoid of phenylalanine and tyrosine have shown that, in the rat, supplementation of the food with $d(+)$-phenylalanine induces growth which is almost, if not quite, as satisfactory as when $l(-)$-phenylalanine is the supplement. This finding has been confirmed by feeding dl- and $l(-)$-phenylalanine, at varying dietary levels, to a relatively large number of animals. The requirement for maximum gains was not detectably greater in the case of the racemic compound than when the natural isomer was used.

Under the conditions specified, and when liberal amounts of the non-essential amino acids, other than tyrosine, and all of the essentials are included in the food, the minimum level of dietary phenylalanine which is capable of inducing maximum increases in weight is *approximately* 0.9 per cent.

BIBLIOGRAPHY

1. Berg, C. P., *J. Biol. Chem.*, **104**, 373 (1934).
2. Cox, G. J., and Berg, C. P., *J. Biol. Chem.*, **107**, 497 (1934).
3. Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 1 (1907).
4. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1937–38).
5. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
6. Rose, W. C., *Science*, **86**, 298 (1937).
7. Rose, W. C., *Proc. Inst. Med. Chicago*, **12**, 98 (1938).
8. du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98**, 295 (1932).
9. du Vigneaud, V., Sealock, R. R., and Van Etten, C., *J. Biol. Chem.*, **98**, 565 (1932).
10. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **141**, 375 (1941).

THE INTERRELATIONSHIP OF DIETARY, SERUM, WHITE BLOOD CELL, AND TOTAL BODY ASCORBIC ACID

By OLIVER H. LOWRY, OTTO A. BESSEY, MARY JANE BROCK, AND JEANNE A. LOPEZ

(*From the Division of Nutrition and Physiology, The Public Health Research Institute of the City of New York, Inc., New York*)

The white blood cells are living, actively metabolizing cells which are accessible for analysis. If the ascorbic acid concentration of these cells could be shown to be a valid index of the ascorbic acid level of the body as a whole, white cell assays should be a valuable means of determining the status of the individual in regard to this vitamin. The analysis of blood or urine yields information which is of value chiefly if it may be interpreted in terms of function of the organism, or in terms of the tissue concentrations on which functional activity surely depends. The establishment of the interrelationships among serum, urine, white cell, and tissue concentrations should enhance the value of existing ascorbic acid data on serum and urine.

Stephens and Hawley (1) and Butler and Cushman (2) found that white blood cells and blood platelets are rich in ascorbic acid in normal persons. The latter investigators found the level very low in scorbutic patients. (White blood cells and platelets contain essentially the same concentration of ascorbic acid and are usually measured together. They will be referred to collectively, hereinafter, as white cells.) Crandon *et al.* (3) observed that on an ascorbic acid-free diet the serum level of the vitamin fell almost to zero, after which the white cell level slowly fell and approached zero at the time frank signs of scurvy appeared. These data have created the impression that the white cells, and hence presumably the other tissues, contain undiminished amounts of the vitamin as long as ascorbic acid is demonstrable in the serum. Pijoan and Lozner (4) have presented data in support of this conclusion. Such data do not, however, concur with the repeated observation that considerable amounts of ascorbic acid may be retained (in a load test) by persons with low but definite serum ascorbic acid concentrations (5).

Clearly more information on the relationship between intake and serum and white cell levels is desirable.

An exceptional opportunity for making the desired comparison was offered by an experiment conducted by the Royal Canadian Air Force. Over 100 vigorous RCAF personnel were maintained for 8 months on

controlled ascorbic acid intakes.   Monthly serum ascorbic acid determinations were made by the primary investigators.   Group Captain Frederick F. Tisdall very kindly invited this laboratory to obtain blood samples from these subjects for determination of white cells and serum ascorbic acid.   The development of a rapid procedure for measuring white cell ascorbic acid concentrations on finger blood (0.1 ml.) made such determinations feasible.[1]

In addition to observing the effect of different ascorbic acid intakes on the serum and white cell concentrations, the extent of the tissue depletion was measured by determining the amount of ascorbic acid necessary to restore the concentration in the white cell and in the body as a whole to maximal values.   These measurements permitted an evaluation of the white cell as an index of the total ascorbic acid in the body.

Materials and Methods

The details of the RCAF experiment have been described by Linghorne et al. (6).   In brief, the personnel were divided into four groups.   Group I received a palatable basal diet which was repeatedly shown by analysis to contain an average of 8 mg. of ascorbic acid per day.   Group II received the same diet plus a raw cabbage supplement containing (by analysis) 15 mg. of ascorbic acid (average total 23 mg.).   Group III received the basal diet plus 70 mg. per day of synthetic ascorbic acid (total 78 mg.).   Group IV received the regular station ration which furnished an average of 78 mg. of ascorbic acid per day.

Ascorbic acid was measured in duplicate on (a) 10 c.mm. serum samples (7), (b) the white blood cells plus platelets obtained from 0.1 ml. of blood,[1] and (c) 0.025 to 0.1 ml. aliquots of 24 hour urine specimens.   The procedure for urine was essentially the same as that used for serum except that larger volumes were employed.   The white cell method has since been improved and is reported elsewhere.[1]

Results

Groups I and II receiving 8 and 23 mg. of ascorbic acid per day had average white blood cell values only about half as great as did Groups III and IV, receiving 78 mg. per day (Fig. 1).   The average serum value for both Groups I and II was the same (0.2 mg. per cent).   No serum values of zero were encountered and all but two samples were 0.1 mg. per cent or over.   As was found for the serum values, there was no statistically significant difference between the white cell averages for Groups I and II. There was also no significant difference between the serum and white cell averages for Group III receiving synthetic ascorbic acid and Group IV receiving the vitamin in natural form (total intakes 78 mg. daily).

[1] Bessey, O. A., Brock, M. J., and Lowry, O. H., in preparation.

On inspection of the graph in Fig. 1 it will be seen that a definite relationship exists between ascorbic acid concentration in serum and white cells. Of the thirty-nine subjects with serum values greater than 0.4 mg. per cent, only one white cell value was less than 20 mg. per cent, whereas out of the forty-two with serum ascorbic acid less than 0.3 mg. per cent, only in one subject did the white cell level exceed 20 mg. per cent. In the intermediate zone of serum values (0.3 to 0.4 mg. per cent) both

Group		Intake mg./day	W.B.C. mg./100ml.	Serum mg./100ml.
I	o	8	11.9±0.4*	0.18±0.01
II	+	23	12.9±0.4	0.20±0.01
III	•	78	24.2±0.6	0.79±0.04
IV	□	78	23.7±0.6	0.73±0.05
			*P.E.M.	
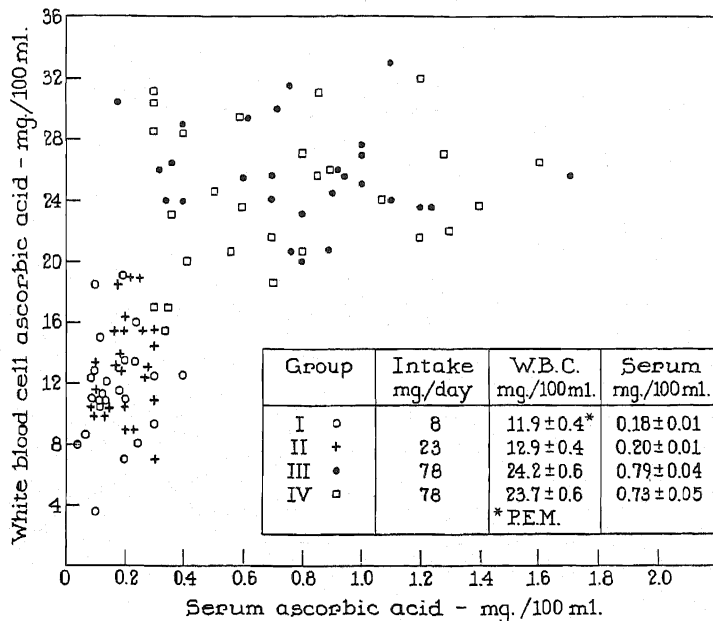
FIG. 1. Comparison of the ascorbic acid concentration in serum and white blood cells of a group of 103 subjects maintained for 8 months on 8, 23, or 78 mg. of ascorbic acid daily.

high and low white cell levels were encountered. The relation between intake and serum and white cell levels is evident.

At the end of 8 months on the rigidly controlled ascorbic acid intakes, thirty subjects, chiefly from Group I (8 mg. per day), were given large amounts of ascorbic acid and the course of realimentation was studied. Each day for 4 days 500, 1000, or 2000 mg. of ascorbic acid were given by mouth in 8 or 10 hourly doses. 24 hour urine samples were collected and each morning, before any ascorbic acid was received, white cell and serum samples were collected.

The data summarized in Figs. 2 and 3 show that for the subjects who had been receiving 8 mg. of ascorbic acid per day recovery of both serum

and white cell levels was nearly complete in 1 day with 2000 mg. of ascorbic acid per day (four subjects), in 2 days with 1000 mg. per day (six subjects), and in 3 or 4 days with 500 mg. per day (four subjects). Thus both serum and white cell values can respond with great rapidity to a sufficiently large dose of the vitamin. The changes in ascorbic acid levels in white cells *versus* serum follow essentially the same pathway (but at different rates) whether the ascorbic acid dose is 500, 1000, or 2000 mg. per day (Fig. 3).
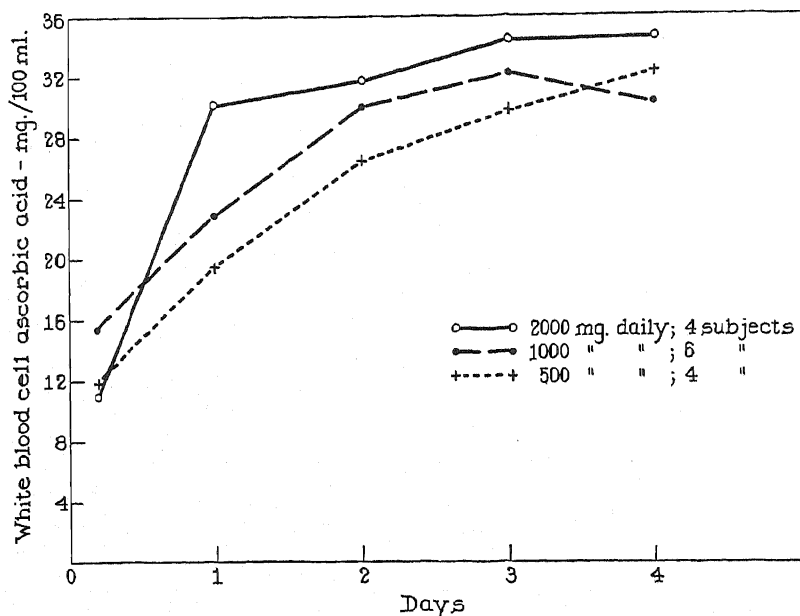
FIG. 2. Summary of changes in ascorbic acid content of white blood cells upon realimentation of subjects previously maintained on low ascorbic acid intakes.

Furthermore, this pathway nearly coincides with the curve obtainable from Fig. 1 for static values of white cell and serum ascorbic acid attained by the ingestion of different amounts of ascorbic acid. This suggests that the white cells and serum are in equilibrium with each other under the conditions of the experiment.

The serum and white cell response of six subjects (data not shown) who had been receiving 23 mg. of ascorbic acid per day was essentially the same as for those who had been receiving 8 mg. Although the average serum levels of a control group of four subjects (from Group III, which had been regularly receiving 78 mg. of ascorbic acid daily) were raised by the large test doses, the white cell levels were not further raised; on the contrary, they decreased slightly (about 15 per cent) during the 4 days of the experiment. This suggests that factors other than ascorbic acid intake may at

least to a small degree affect the ascorbic acid level of white blood cells. Similar unexplained changes have been encountered by others (Wilson and Lubschez (8)).

The urinary excretion (Table I) was uniformly low during the first 24 hours except in the control group (No. III). It is surprising that, even when 2000 mg. of ascorbic acid were ingested, only 0.5 per cent of the dose was lost in the urine. On subsequent days the excretion rose to 75 to 90 per cent of the intake. An estimate of the ascorbic acid retention was
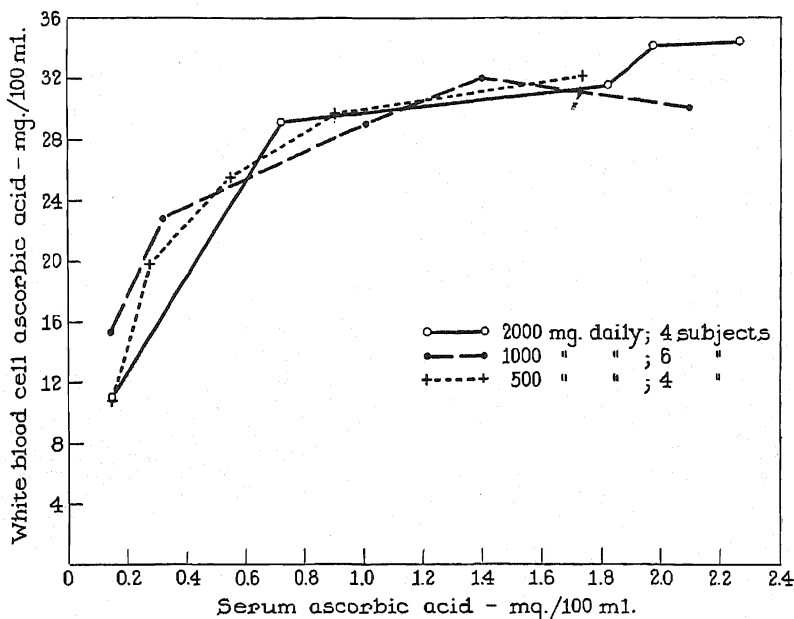
FIG. 3. Comparison of the ascorbic acid content of white blood cells and serum during realimentation of subjects previously maintained on low ascorbic acid intakes.

made with the following assumptions: (a) that the ingested vitamin was in part retained, in part destroyed, and in part excreted in the urine; (b) that during the last 24 hour period the control group (No. III), receiving 500 mg., and the groups receiving 1000 and 2000 mg. of ascorbic acid were no longer retaining ascorbic acid, in which case the destruction in the body would be equal to the difference between intake and output; (c) that this destruction was the same on all 4 days, and was equal in the three groups receiving 500 mg. per day.

If these assumptions are valid, the retention each day may be calculated by subtracting the ascorbic acid excreted that day from the amount excreted at a time when no retention was taking place. For example, the

group receiving 1000 mg. excreted 1, 125, 495, and 767 mg. on the 4 successive days; the calculated retention was, therefore, 766, 642, 272, and 0 mg. respectively, or a total of 1680 mg.   This is presumably a lower limit, since there is quite possibly less destruction of ascorbic acid during the 1st day or 2.   Considering this uncertainty, the agreement in retention with

TABLE I

Average Daily Urinary Excretion and Total Retention of Ascorbic Acid during Realimentation

Group No.	Previous intake	Test dose	Excretion				Retention
			1 day	2 days	3 days	4 days	
	mg. per day	*mg. per day*	*mg. per day*	*mg. per day*	*mg. per day*	*mg. per day*	*mg.*
I	8	500	2	22	72	167	1625
II	23	500	1	13	77	260	1537
III	78	500	154	371	470	474	419
I	8	1000	1	125	495	767	1680
I	8	2000	11	980	1403	1539	2223

TABLE II

Comparison of Retention of Ascorbic Acid to Increase in White Blood Cell Ascorbic Acid

Group No.	Previous intake	Test dose		Change as per cent of maximal change	
				After 18 hrs.	After 42 hrs.
	mg. per day	*mg. per day*			
I	8	500	Total body*	29	57
			White blood cells†	40	68
I	8	1000	Total body	46	87
			White blood cells	44	81
I	8	2000	Total body	69	
			White blood cells	78	
II	23	500	Total body	31	60
			White blood cells	33	83

* Retention as per cent of total retention.
† Increase in concentration as per cent of total increase in concentration.

different dosages is satisfactory.   The average retention for the fourteen subjects in Group I was 1844 mg.   Group II, which had been initially receiving 23 mg. of ascorbic acid per day, retained nearly as much ascorbic acid as Group I (initially receiving 8 mg. per day), whereas the control group retained only about one-fourth as much.

In reference to the question whether the white blood cell ascorbic acid level can serve as an index of general tissue levels of ascorbic acid, it is

useful to correlate the total daily retention of ascorbic acid by the body as a whole with the rate at which the white cells return to normal (Table II). Total retention is recorded as per cent of the ultimate total retention, and the white blood cell changes are recorded as per cent of the total change during the 4 days; *i.e.*, if the initial, 1st day, and 4th day white cell ascorbic acid values were 12, 21, and 30 mg. per cent, the change after 1 day would be recorded as 100 × 9/18 or 50 per cent. It will be seen (Table II) that the white cell recovery parallels the ascorbic acid retention within the limits of experimental variations expected for this type of study. This suggests that the white blood cells are actually a valid measure of the level of the body content of ascorbic acid. If this is true, the total body ascorbic acid may be calculated: Since the white cell ascorbic acid concentration approximately doubled during the retention of 1800 mg. of ascorbic acid, the final body content of ascorbic acid is presumably twice 1800 mg. or nearly 4 gm.

DISCUSSION

It seems permissible to conclude (1) that with the ingestion of 8 to 23 mg. of ascorbic acid daily by adults the white blood cell ascorbic acid is maintained at only about half the concentration attained with intakes of 78 mg. per day; (2) that with 8 to 23 mg. of ascorbic acid per day a tissue deficit from the maximal attainable concentration amounting to nearly 2 gm. is encountered; (3) that with white cell ascorbic acid values half of normal, serum ascorbic acid levels are not necessarily zero but lie rather in the concentration range of 0.1 to 0.4 mg. per cent; (4) that the white cell level appears to be an index of the total body level of ascorbic acid; and (5) that given an opportunity, through a sufficiently large intake of ascorbic acid, the white cells and serum levels will return to normal within 24 hours, with a concomitant retention of very large amounts of ascorbic acid by the body.

An important question to public health is what tissue concentration is necessary for optimal ascorbic acid nutrition. In this study the control group receiving 78 mg. per day was approximately 90 per cent saturated (400 mg. retention with a calculated 4 gm. of ascorbic acid content at saturation). Groups I and II with 8 and 23 mg. of ascorbic acid per day were 50 or 60 per cent saturated. Careful studies of the gums of these subjects by Linghorne *et al.* (6) revealed a clearly defined handicap to the gingival tissue in Groups I and II. This tissue is continually exposed to mechanical trauma and bacterial invasion. It is precisely such conditions which are likely to expose handicaps attributable to suboptimal nutritional states. These findings suggest, therefore, that with somewhere between 60 and 90 per cent tissue saturation handicaps attributable to ascorbic acid deficiency become demonstrable.

The degree of tissue undersaturation appears to be measured in a general way by the ascorbic acid concentration in the white blood cells. It will be frequently desirable, however, to have a more direct and sensitive measure of the tissue deficit, particularly when the depletion is not great. The usual saturation tests are not as sensitive a measure of this deficit as they could be. Common practice is to give a single fairly large dose, perhaps 200 to 500 mg., and to measure the amount of ascorbic acid which appears in the urine. In any case, but particularly if this dose is given by vein, the vascular system is flooded with ascorbic acid and the amount excreted depends on how well the kidney competes with the rest of the body for the vitamin. When given by mouth, the serum level and, hence the urinary excretion, will depend in part on the rate of absorption. Thus the information obtained gives relative but not absolute information concerning the tissue deficit. An alternative procedure is suggested by the experiments reported here: A total dose should be given which is somewhat larger than the expected deficit; e.g., 750 to 1000 mg. for persons receiving over 50 mg. per day, and for persons thought to be seriously depleted, 2 or 3 gm. The ascorbic acid should be given in many divided doses spread out during the day, and all the urine excreted during a 24 hour period, commencing with the first dose, should be collected. With allowance for an estimated 25 per cent destruction, the ascorbic acid retention can be estimated. This procedure is more involved than the ordinary tolerance test, but it has the virtue of giving a measure of the actual tissue depletion. Hathaway and Meyer (9) have used this type of test in studies with children, but they used much smaller doses administered over a much longer period of time. The relatively small amount of destruction of ascorbic acid by the body in comparison to that of thiamine, riboflavin, and niacin creates an especially favorable opportunity for the measurement of tissue depletion by this method (10).

SUMMARY

1. White blood cell and serum ascorbic acid concentrations were measured in subjects who had received standardized diets for 8 months containing 8, 23, and 78 mg. of ascorbic acid per day. With 8 and 23 mg. of the vitamin per day the white cells averaged about 12 mg. per cent of ascorbic acid compared to 25 mg. per cent of ascorbic acid for subjects receiving 78 mg. per day. The serum levels were not zero even with the low intakes and low white cell levels, averaging 0.2 mg. per cent for those receiving 8 and 23 mg. of ascorbic acid per day.

2. After 8 months on a diet containing 8 mg. of ascorbic acid per day an average of about 1800 mg. of ascorbic acid was retained following the ingestion of large amounts of ascorbic acid.

3. Since the increase in white cell ascorbic acid concentration paralleled the retention of ascorbic acid, the white blood cells appear to be a valid index of the total body concentration of ascorbic acid. This leads to the conclusion that the normal adult contains nearly 4 gm. of ascorbic acid.

BIBLIOGRAPHY

1. Stephens, D. J., and Hawley, E. E., *J. Biol. Chem.*, **115**, 653 (1936).
2. Butler, A. M., and Cushman, M., *J. Clin. Invest.*, **19**, 459 (1940).
3. Crandon, J. H., Lund, C. C., and Dill, D. B., *New England J. Med.*, **223**, 353 (1940).
4. Pijoan, M., and Lozner, E. L., *Bull. Johns Hopkins Hosp.*, **75**, 303 (1944).
5. Stotz, E., Shinners, B. M., and Chittick, R. A., *J. Lab. and Clin. Med.*, **27**, 518 (1942).
6. Linghorne, W. J., McIntosh, W. G., Tice, J. W., Tisdall, F. F., McCreary, J. F., Drake, T. G. H., Greaves, A. V., and Johnstone, W. M., *Canad. Med. Assn. J.*, **54**, 106 (1946).
7. Lowry, O. H., Lopez, J. A., and Bessey, O. A., *J. Biol. Chem.*, **160**, 609 (1945).
8. Wilson, M. G., and Lubschez, R., *J. Clin. Invest.*, **25**, 428 (1946).
9. Hathaway, M. L., and Meyer, F. L., *J. Nutr.*, **21**, 503 (1941).
10. Melnick, D., Hochberg, M., and Oser, B. L., *J. Nutr.*, **30**, 67 (1945).

DETERMINATION OF CARBON MONOXIDE IN BLOOD AND OF TOTAL AND ACTIVE HEMOGLOBIN BY CARBON MONOXIDE CAPACITY. INACTIVE HEMOGLOBIN AND METHEMO- GLOBIN CONTENTS OF NORMAL HUMAN BLOOD

By DONALD D. VAN SLYKE, ALMA HILLER, JAMES R. WEISIGER, AND WALTER O. CRUZ

WITH THE TECHNICAL ASSISTANCE OF JOHN PLAZIN

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

The occurrence in normal human blood either of methemoglobin, or of a form of "inactive Hb" which behaves like methemoglobin in containing trivalent Fe and in lacking the power to combine reversibly with O_2 and CO until the Fe^{+++} is reduced to Fe^{++}, has been both affirmed and denied in the recent literature.[1] Until a few years ago all the hemoglobin in normal human blood was assumed to be in the "active" form, capable of reversibly combining with O_2 and CO. Methemoglobin could not be detected by spectroscopic examination, and the classic studies of Peters (6) in Barcroft's laboratory showed an almost exact ratio of 1 atom of Fe per molecule of O_2 capacity in laked blood. However, since quantitative gasometric methods for the estimation of inactive Hb and photometric methods for methemoglobin have been developed, widely varying amounts of both in normal human blood have been reported.

The gasometric methods for inactive Hb have been based on a principle introduced by Nicloux and Fontes (1) whereby, by means of a reducing agent, the inactive Hb, with Fe^{+++}, is reduced to active Hb, with Fe^{++}, and the resultant increase in CO or O_2 binding capacity is determined as the measure of the inactive Hb. Nicloux and Fontes used $Na_2S_2O_4$ as reducing

---

[1] *Nomenclature*—In the present paper the term "active Hb" will be used to indicate hemoglobin capable of combining reversibly with O_2 or CO; "inactive Hb" to indicate hemoglobin derivatives that do not combine reversibly with O_2 or CO, but can be converted into active Hb by the reducing agents that convert ferrihemoglobin into ferrohemoglobin. "Methemoglobin" is used to indicate inactive Hb which, in addition to the ability to be converted into active Hb by reducing agents, also shows on treatment with cyanide the changes in optical density that are characteristic of methemoglobin. In earlier literature it was assumed that all inactive Hb, as defined above, was methemoglobin, and methods for gasometric determination of inactive Hb were presented as methods for methemoglobin (1-3). In more recent papers (4, 5) the possibility that inactive hemoglobin derivatives other than methemoglobin may occur in blood has led to use of the term "inactive hemoglobin" to indicate the substance or substances that are measured by the increase in CO or O_2 capacity caused by treatment of blood with $Na_2S_2O_4$ or Ti^{+++}.

agent, and the increase in CO capacity as a measure of inactive Hb. Van Slyke (2), using Nicloux' reagents and the Van Slyke-Neill (7) manometric apparatus for determining the CO capacity of the blood, developed a method which was later improved by Van Slyke and Hiller (3, 8) and Horvath and Roughton (9). The first considerable series of measurements in normal human blood by the method (8) was published by Ammundsen (4), who found in 53 subjects that the inactive Hb varied from 0 to 12 per cent of the total Hb, the mean being 3 per cent. In the blood drawn from a single subject on ten different dates the range was found to be about as great as in the entire series of 53 subjects. Roughton, Darling, and Root (5) in determinations on twenty-six blood specimens from four different subjects found practically the same mean inactive Hb, 2.8 per cent of the total Hb, but a narrower range, 0.7 to 5 per cent. However, Kallner (10, 10a), also using the $Na_2S_2O_4$-CO method, was doubtful that any inactive Hb exists in normal human blood.

Conant, Scott, and Douglass (11) showed that hematin when reduced by $Na_2S_2O_4$ combines reversibly like Hb with CO, so that the inactive Hb measured by the $Na_2S_2O_4$-CO method would include, besides methemoglobin, any hematin that might be present, and presumably any Fe^{+++} compounds intermediate between methemoglobin and hematin. As a procedure more specific for methemoglobin, Conant, Scott, and Douglass reduced the Fe^{+++} to Fe^{++} with titanous tartrate, instead of $Na_2S_2O_4$, and measured the resultant increase in O_2 capacity, instead of CO capacity. (O_2, unlike CO, does not combine reversibly with hematin under the conditions of the analyses. Ti^{+++} was substituted for $Na_2S_2O_4$ as reducing agent because the presence of $Na_2S_2O_4$ interferes with O_2 capacity determinations.) Conant, Scott, and Douglass did not give data indicating whether any of the hypothetical Hb derivatives, capable of combining with CO but not O_2, exist in blood, and inactive Hb values for normal human blood obtained by Ramsay (12) with Ti^{+++} and O_2 are not markedly different from those obtained by Ammundsen and by Roughton, Darling, and Root with $Na_2S_2O_4$ and CO. In thirty-eight subjects Ramsay found inactive Hb, varying from 0 to 7 per cent of total Hb, the mean being 1.9.

Recent photometric methods for methemoglobin depend on the decrease of transmission of light of wave-length about 6350 (13–15) or 8000 (16) Ångströms that occurs when KCN is added to a solution of methemoglobin; the cyanide changes the brownish methemoglobin to the red cyanhemoglobin, but does not affect the active Hb. Methemoglobin values in normal human blood reported by this procedure vary, as is indicated by Table I.

The results in the literature leave uncertainty, both as to the identity of the inactive Hb measured by the Nicloux principle with the substance

measured as methemoglobin by the photometric cyanide method, and as to the concentration range of either substance in freshly drawn normal human blood. No author appears to have made simultaneous measurements by both procedures. In most of the papers the length of time the blood samples stood before the analyses were made is not exactly stated, so that the effects of changes *in vitro* are uncertain.

TABLE I

Methemoglobin and Inactive Hb in Normal Human Blood; Data in Literature

Author	No. of subjects	No. of blood specimens	Method used	Per cent of total Hb		
				Minimum	Maximum	Mean
Inactive Hb						
Ammundsen (4) (1937, 1939, 1941)...............	53	63	$Na_2S_2O_4 + CO$ gasometric	0	12.0	3.0
Kallner (10, *a*) (1942)	20	20	" "	−1.2	1.2	−0.2
Roughton, Darling, and Root (5) (1944)..........	4	26	" "	0.7	5.0	2.8
Kallner (10) (1945)........	4	4	" "	−1.5	0.5	−0.5
Ramsay (12) (1944)........	38	38	$Ti^{+++} + O_2$ gasometric	−0.5	7.0	1.9
Methemoglobin						
Havemann *et al.* (14) (1939)...................			KCN photometric		8	
Schmid-Burgk (17)* (1940).	20	20	" "	1.1	2.4	1.7
Paul and Kemp (18)† (1944)...................	20	20	" "	0.1	0.8	0.4

* The results of Schmid-Burgk are reported in papers by Heubner (17).

† In 100 miscellaneous hospital patients, selected at random except that those receiving sulfonamides or other recognized methemoglobin formers were excluded, results were about the same as in normals, except that 2 out of the 100 were higher, about 2 and 3 per cent of total Hb, respectively.

In the present paper results are reported of simultaneous determinations made by both procedures at intervals beginning 2 to 3 minutes after the blood was drawn. For methemoglobin the cyanide procedure developed by Horecker and Brackett (16) is used. For the inactive hemoglobin Nicloux' principle of measuring the increase in CO binding power caused by treatment with $Na_2S_2O_4$ is employed. Since the increases measured were of the order of 1 per cent, maximal obtainable analytical accuracy was essential. The procedure used for the CO capacity determinations

is that of Van Slyke and Hiller (3, 8), with improvements introduced by Horvath and Roughton (9), and with other modifications for precision that have developed in this laboratory since the original method (3, 8) was published.

It cannot be stated with certainty that the cyanide reaction applied to blood is completely specific for methemoglobin, but the results in this paper indicate that, if the cyanide reaction does measure any normal blood constituent other than methemoglobin, the amount of such constituent is slight.

DETERMINATION OF BLOOD CO CONTENT AND OF ACTIVE AND TOTAL HEMOGLOBIN BY CO CAPACITY

The carbon monoxide procedures for blood CO content, total hemoglobin, and active hemoglobin, as developed, are described with the modifications necessary for blood samples varying from 2 to 0.1 ml. The technique is given in detail necessary for precision.

Apparatus

The *Van Slyke-Neill manometric apparatus* (7).

The *special Hempel pipette*, with 3-way cock, described by Van Slyke and Hiller (3).

The *apparatus for generating and storing CO* gas described by Van Slyke and Hiller (3).

Calibrated glass spoons for approximate measurement of pulverized $Na_2S_2O_4$, as used by Van Slyke and Folch ((19), Fig. 4, *A*).

The spoon may be made by flattening one end of a rod to a disk of about 6 mm. in diameter, and fusing a tube of 3 mm. bore to the disk. The tube is then cut off so that a cup about 4 mm. deep is made. The cup is ground down on a carborundum wheel until its capacity is 35 ± 5 mg. of powdered $Na_2S_2O_4$. One such spoon suffices for analyses of samples of either 1 or 2 ml. of blood. For analyses of samples of 0.5 and of 0.1 or 0.2 ml., smaller spoons are made to deliver 18 ± 2 mg. and 7 ± 1 mg. portions of hyposulfite respectively.

A simpler procedure devised by D. A. MacFadyen (personal communication) is to bend a piece of tubing at right angles, cut one arm off at about 6 mm. from the bend, and fill the bend with high melting paraffin, the level of which is adjusted to give the desired measuring capacity.

Syringe modified to store and deliver air-free solutions. The syringe, shown in Fig. 1, is made by fusing a 20 ml. graduated Pyrex Becton-Dickinson syringe to a Pyrex glass stop-cock with 1 to 1.5 mm. bore and a stem long enough (15 to 20 cm.) to reach to the bottom of a 250 ml. suction flask. The tip of the stem is tapered so that it can be equipped with a rubber ring which fits into the bottom of the cup of the gas chamber.

After fusing the stop-cock to the syringe it may be necessary to wait 24 hours for the barrel to contract exactly to its former bore and fit the plunger.

Reagents

Sodium hyposulfite ($Na_2S_2O_4$), finely pulverized.

Saponin-borax solution. 1 gm. of saponin and 3 gm. of borax, $Na_2B_4O_7 \cdot 10H_2O$, are dissolved in 100 ml. of water. Add 0.1 ml. of caprylic alcohol as a preservative and shake to saturate the solution.
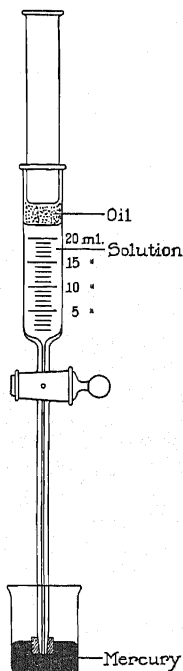
FIG. 1. Syringe for storing and delivering air-free solution

Potassium ferricyanide solution. 32 gm. of $K_3Fe(CN)_6$ per 100 ml.

Acetate buffer to produce pH approximately 6. 75 gm. of sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, are dissolved in 100 ml. of water and 15 ml. of glacial acetic acid are added.

Sodium hydroxide. 1 N solution, deaerated by extraction in the evacuated Van Slyke-Neill chamber and stored over mercury.

Caprylic alcohol.

Carbon monoxide gas. This is prepared from H_2SO_4 and formic acid and stored as described by Van Slyke and Hiller (3). About 5 per cent of

NaOH is added to the water over which the CO is stored in order to absorb a small amount of CO_2 which may accompany the CO.

Ferricyanide in acetate buffer. 5 ml. of the acetate buffer solution and 15 ml. of the 32 per cent ferricyanide solution are mixed on the day of the analyses and either saturated with air or deaerated.

The *air-saturated solution* may be used for hemoglobin determinations when maximal precision is not imperative. The air-saturated solution is prepared by mixing, on the day of analysis, the 5 ml. of acetate buffer and 15 ml. of 32 per cent ferricyanide, and bringing the mixture completely to equilibrium with the atmosphere, with respect to gas content. The equilibration may be accomplished by placing the solution in a half liter flask and rotating the liquid about the walls of the flask for a few minutes, or by bubbling a rapid stream of air through the solution. In either case the temperature of the solution is taken at the end of the saturation, to make sure that it is within 0.5° of that of the room. The aerated solution is drawn up into a 25 ml. burette, provided with a capillary stem that is fitted with a rubber ring at the tip and that is long enough to reach to the bottom of the cup of the manometric chamber. The extent to which the air content of the solution may vary on different days is indicated by the c corrections of the top row of figures in Table V. In the burette the air content of the solution will not change significantly during the day, unless unusual variations occur in temperature or barometer.

The *deaerated solution* is required when precision is needed, such as is required for the determination of inactive Hb from the difference between total and active Hb. The procedure generally used to prepare air-free reagents, *viz.* to extract the air in the Van Slyke-Neill chamber and store the solution over mercury, cannot be used here, because contact with mercury reduces the ferricyanide to ferrocyanide. The procedure adopted is to deaerate the solution in an evacuated flask, and then draw it up into a syringe of which the dead space is filled with deaerated mineral oil (Fig. 1). Between analyses the syringe is suspended with its tip dipping into mercury.

The syringe is charged with air-free oil and solution as follows: Deaerate 50 to 60 ml. of mineral oil by placing it in a 250 ml. suction flask, evacuating the flask, warming the bottom in a bath at 60–70° and occasionally whirling the solution about the flask, until evolution of bubbles ceases. For the evacuation a water pump suffices (15 to 20 mm.), but deaeration is more rapid with a mechanical vacuum pump, lowering the pressure to about 1 mm. After the oil is deaerated, open the flask, immediately draw about 5 ml. of the oil from the bottom layer into the syringe, and eject the air bubble.

Under the oil remaining in the suction flask run 5 ml. of the acetate buffer solution and 15 ml. of the 32 per cent ferricyanide solution. Evacuate with a water pump and deaerate as described for the oil. When the evacuated flask is warmed, the aqueous solution boils and the vapor accelerates removal of the last portions of air. After the solution has boiled *in vacuo* for a few minutes, close the rubber tube at the outlet with a screw clamp and let the evacuated flask stand for 5 or 10 minutes, until the aqueous solution settles out of the emulsion. Then open the flask and immediately draw the solution into the syringe under the oil. Hang the syringe with its tip dipping under mercury.

Procedures for Total Hb, Active Hb, and CO Content of Blood

The procedure for *total Hb* consists of (1) reducing the ferrihemoglobin to ferrohemoglobin with $Na_2S_2O_4$, (2) saturating the Hb with CO gas, (3) extracting the uncombined CO and N_2 from the solution, (4) adding ferricyanide to set CO free from HbCO, and (5) extracting and measuring the CO liberated from the HbCO.

For *active Hb*, the procedure is the same as for total Hb except that step (1) is omitted.

For the *CO content of drawn blood* the procedure is the same as for total Hb, except that step (2) is omitted, and pressure readings are taken at 0.5 ml. volume, even when the sample is 2 ml.

The *procedure for total Hb with 1 ml. samples* will be detailed, with indication of the steps that are omitted when active Hb or CO content is determined. For samples of 2, 0.5, 0.2, or 0.1 ml. the procedure is the same as for 1 ml., except that different amounts of reagents are used, as indicated in Table II, and that when 2 ml. samples are used for total or active Hb the pressure readings are made with the CO gas at 2.0 ml. rather than 0.5 ml. volume.

Transfer of Blood Sample to Chamber of Gas Apparatus—2 drops of caprylic alcohol are placed in the cup of the chamber, and most of the alcohol is drawn down into the chamber, enough being left above the cock to fill the capillary between the cock and the cup. 3 ml. of the saponin-borax solution are placed in the cup, and about 0.5 ml. is drawn down into the chamber. The admission of solution, and of the blood later, is controlled by the cock leading to the mercury bulb, the cock at the top of the chamber being left open. If the blood has been standing, even for a minute, it is thoroughly mixed, and the sample is drawn into a 1 ml. pipette (without stop-cock), calibrated for complete delivery, and fitted at the tip with a rubber ring. The tip of the pipette containing the sample is pressed through the solution in the cup above the chamber and the rubber ring is fitted into the bottom of the cup as shown in Fig. 2. The blood is then delivered at a slow, even rate into the chamber, the rate of delivery being regulated by the cock leading to the mercury leveling bulb. About 2 minutes should be taken to empty the pipette, in order to obtain the most exact delivery of the blood sample. The delivery is continued until all the blood has left the pipette, and a slight bubble of air has followed the blood into the capillary beneath the cup. The cock at the top of the chamber is then closed, and the bubble at the top of the capillary is dislodged by means of a fine wire dipped into caprylic alcohol. A little of the saponin-borax solution is then run into the chamber to wash the blood out of the capillary.

Addition of $Na_2S_2O_4$ (Omitted in Determination of Active Hb)—To the

saponin-borax solution remaining in the cup above the chamber 35 ± 5 mg. of pulverized $Na_2S_2O_4$ are added from the calibrated glass spoon. At once after the hyposulfite is added, it is dissolved by stirring for a few seconds with a slender rod, and the borax-hyposulfite solution is immediately drawn down into the chamber, before atmospheric O_2 can oxidize the hyposulfite. Enough solution is left above the cock to fill the capillary. About 0.5 ml. of mercury is poured into the cup to provide a seal for the tube from which the CO gas is next delivered.
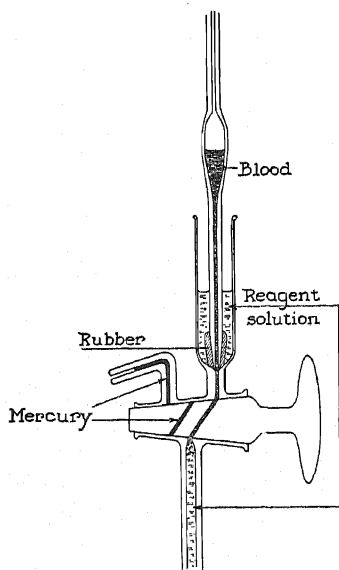
Fig. 2. Delivery of blood sample into chamber of Van Slyke-Neill apparatus

Delivery of CO into Chamber of Gas Apparatus (Omitted in Determination of CO Content of Drawn Blood)—From the modified Hempel pipette approximately 10 ml. of CO (volume at atmospheric pressure) are delivered into the chamber, as described by Van Slyke and Hiller (3). The measurement is sufficiently accurate if it is within ±10 per cent. The CO may be measured by volume at approximately atmospheric pressure by admitting the CO until 10 ml. are present, as measured by a mark made on the water jacket of the chamber. Or the CO may be measured by pressure, as described by Van Slyke and Hiller (3): the mercury meniscus is lowered to the 50 ml. mark, and alternately CO is admitted at the top of the chamber and mercury at the bottom, the mercury meniscus being kept near the 50 ml. mark, until the pressure indicated by the manometer has risen 150 mm.

Saturation of Hemoglobin with CO (Omitted in Determination of CO Content of Drawn Blood)—The 0.5 ml. of mercury in the cup is run into the chamber, enough being left behind to fill the capillary above the cock. The mercury in the chamber is lowered to the 50 ml. mark, and the chamber is shaken for 1.5 to 2 minutes. It is desirable not to shake longer, because contact for as long as 5 minutes at this stage between the hemoglobin and the alkaline solution appears to cause the beginning of denaturation; the amount of HbCO after 5 minutes saturation has been found slightly less (a few hundredths of a volume per cent of CO) than after 1 or 2 minutes. During the shaking the chamber should not be exposed to direct sunlight. After the shaking is complete, the CO gas is ejected from the chamber.[2]

With the above treatment the cells have been laked by the saponin, the O_2 originally present in the blood has been reduced by hyposulfite, any ferrihemoglobin present has been reduced to ferrohemoglobin, and, together with the ferrohemoglobin originally present, has been combined with CO to form HbCO. Also, the greater part of the N_2 has been extracted from the solution.

Extraction of Uncombined CO and Residual N_2 from Solution—The cock at the top of the chamber is sealed with mercury, the mercury in the chamber is lowered to the 50 ml. mark, and the evacuated chamber is shaken for 2 minutes at the rate of 300 to 400 oscillations per minute. The solution must be protected from direct sunlight or bright diffuse light, as light decreases the affinity of Hb for CO. If the room is at all bright, the chamber is covered with a black cloth during the extraction. The extracted gas is ejected, without ejection of any of the solution,[2] and the cock is again sealed with mercury. The only gases now left in solution are CO in the form of HbCO, and CO_2 chiefly as HCO_3^- and $CO_3^=$. As shown by Horvath and Roughton (9), in the alkaline borax solution, in the dark, the affinity of Hb for CO is so great that no significant part of the CO present as HbCO is lost during the extraction.

Extraction of CO from HbCO—The mercury in the chamber is lowered enough to bring the surface of the aqueous solution down into the broad portion of the chamber. This is done in order to avoid precipitation of methemoglobin on the walls of the upper narrow part of the chamber when the ferricyanide-acetate is next added. In the cup above the chamber are

[2] The following technique serves for ejecting gases without loss of solution. With the cock leading to the leveling bulb open, the mercury in the leveling bulb is brought to the level of the solution meniscus in the chamber, putting the contents of the chamber under slight positive pressure, and the cock leading to the leveling bulb is closed. The bulb is then placed in the ring above the level of the chamber, the cock at the top of the chamber is opened, and the gas is ejected by slowly opening the cock leading to the leveling bulb. The ejection is continued until the aqueous meniscus rises through the cock and fills the capillary between the cock and the cup.

placed about 0.5 ml. of mercury and 1 or 2 ml. of water. The syringe containing the deaerated ferricyanide-acetate solution (or the burette if an air-saturated solution is used) is held with the tip above the water in the cup, and a drop of the solution is delivered into the water in order to expel any air that may be in the tip. The tip is then pressed through the mercury so that the rubber ring fits against the bottom of the cup, and 1.5 ml. of the ferricyanide-acetate reagent, measured by the graduation on the syringe, is admitted into the chamber, the inflow being regulated by the cock at the top of the chamber. The cock is then sealed with mercury from the cup, and the cup is rinsed with water to remove any ferricyanide left in it. The mercury in the chamber is then lowered to the 50 ml. mark and the chamber is shaken 3 minutes to extract the CO, which is accompanied by CO_2 set free from blood and reagents by the acid in the ferricyanide solution.

Absorption of CO_2 and Measurement of CO—Mercury is admitted from the leveling bulb to the chamber until the gas space is decreased to between 3.5 and 4 ml. 3 ml. of air-free 1 N NaOH are placed in the cup with minimal exposure to air, and the lower 1.5 ml. are at once run into the chamber, without agitation, during 30 to 60 seconds. The gas space previously left in the chamber should be such that the last portion of the added alkali mounts into the narrow tube with the 2 ml. mark. The alkali, if quietly added, forms a clear layer over the hemoglobin solution, permitting accurate reading of the meniscus. After the alkali, a few droplets of mercury are admitted to dislodge alkali solution from the space underneath the stop-cock. 1 minute is allowed for the alkali to finish draining down the wall; the meniscus is then raised to the 0.5 ml. mark, preferably observed with a hand lens, and the reading p_1 is taken on the manometer. The temperature in the water jacket is recorded. The gas is then ejected from the chamber,[2] the solution meniscus is lowered below the 0.5 ml. mark, then raised to the mark, and the manometer reading p_0 is taken, with the chamber free of gases other than water vapor.

Blank Analysis to Determine c Correction—The blank analysis is carried out exactly as the blood analysis, except that water is substituted for the blood. The $p_1 - p_0$ value found in the blank analysis is the c correction. In analyses of 1 ml. blood samples the c value measured, with the gas at 0.5 ml. volume, should not exceed 1.5 mm. in determination of total Hb, or 3.5 mm. in determination of active Hb, if the 1 N NaOH and the acetate-ferricyanide solutions have been kept and used with the precaution prescribed to keep them air-free (see Table V). In analyses of 2 ml. blood samples, c measured with the gas at 2 ml. volume is one-fourth as great as in the analyses of 1 ml. samples.

Washing Chamber after Analyses—It is necessary to remove all particles

of methemoglobin precipitate from the walls of the chamber. If any remain, they are likely to be reduced to active Hb by $Na_2S_2O_4$ added in the next analysis and cause a plus error.

After p_0 has been recorded, without releasing the vacuum admit about 10 ml. of water into the chamber, mix with the blood solution by agitating gently by hand, and then eject the mixture from the chamber. Fill the cup of the chamber with 1 N NaOH, dissolve about 70 mg. of $Na_2S_2O_4$ in the NaOH solution, and admit the latter to the chamber, followed by about 20 ml. of water. Lower the mercury below the 50 ml. mark and shake with the motor until all particles adherent to the wall are dissolved. The

TABLE II

*Conditions for Total Hb Determination in Blood Samples of Different Size**

Volume of blood sample	Caprylic alcohol used	Volume of saponin-borax soluton used	$Na_2S_2O_4$ used*	Volume of CO gas in chamber to saturate Hb†	Volume of ferri-cyanide-acetate solution used	1 N NaOH		Volume of gas when p_1 and p_0 are read
						Measured into cup above chamber	Admitted into chamber	
ml.	*drops*	*ml.*	*mg.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*
2	3	4	35 ± 5	10	1.5	3.0	1.5	2.0
1	2	3	35 ± 5	10	1.5	3.0	1.5	0.5
0.5	1	1.5	18 ± 2	10	1.0	2.0	1.0	0.5
0.1–0.2	1	1.0	7 ± 1	10	0.5	2.0	0.5	0.5

* To determine *active Hb*, omit the $Na_2S_2O_4$. In all other details the procedure is the same as for total Hb.

† To determine *CO content* of blood as drawn, omit the saturation with 10 ml. of CO. Make pressure readings with the gas at 0.5 ml. volume, even when the sample is 2 ml. Other details, including addition of $Na_2S_2O_4$, the same as for total Hb.

$Na_2S_2O_4$ assists by reducing insoluble acid methemoglobin to the more soluble reduced Hb. Eject the solution from the chamber. Wash once with water; then admit 20 ml. of water and 2 ml. of 2 N sulfuric acid and shake, with the mercury surface in the broad part of the chamber somewhat above the 50 ml. mark. The dilute acid cleans the mercury of colloidal material that is likely to contaminate it after the previous washing with alkali. Eject the acid solution, and wash twice with 20 ml. portions of water.

Analysis of Blood Samples of Different Size—The above directions are for analyses of blood samples of 1 ml. For samples of 2, 1, 0.5, 0.2, and 0.1 ml., the volumes of reagents used, and the gas volumes at which p_1 and p_0 are measured, are given in Table II.

Calculation—The pressure, P_{CO}, exerted by the CO gas is

$$P_{CO} = p_1 - p_0 - c$$

where c is the $p_1 - p_0$ value obtained in the blank analysis.

The result in terms of volumes per cent or mM per liter of CO, or gm. of hemoglobin per 100 ml. of blood, is calculated by multiplying P_{CO} by the proper factor in Table III or Table IV.

TABLE III

Factors by which Mm. of P_{CO} are Multiplied to Give Blood CO Content

Temperature	Volumes per cent CO in blood					mM CO per liter blood				
	Sample = 2 ml. S = 7.5 ml. a = 2.0 ml. i = 1.00	Sample = 2 ml. S = 7.5 ml. a = 0.5 ml. i = 1.00	Sample = 1 ml. S = 5.5 ml. a = 0.5 ml. i = 1.00	Sample = 0.5 ml. S = 3 ml. a = 0.5 ml. i = 1.00	Sample = 0.2 ml. S = 1.7 ml. a = 0.5 ml. i = 1.00	Sample = 2 ml. S = 7.5 ml. a = 2.0 ml. i = 1.00	Sample = 2 ml. S = 7.5 ml. a = 0.5 ml. i = 1.00	Sample = 1 ml. S = 5.5 ml. a = 0.5 ml. i = 1.00	Sample = 0.5 ml. S = 3 ml. a = 0.5 ml. i = 1.00	Sample = 0.2 ml. S = 1.7 ml. a = 0.5 ml. i = 1.00
°C.										
15	0.1248	0.03120	0.06240	0.1246	0.3113	0.05569	0.01392	0.02784	0.05558	0.1389
16	43	08	16	41	02	50	87	74	38	84
17	39	0.03097	0.06194	37	0.3091	30	82	64	19	79
18	35	86	72	32	80	10	77	54	0.05499	74
19	30	75	50	28	69	0.05490	72	44	80	69
20	26	63	26	24	57	71	67	34	60	64
21	22	53	06	19	47	51	62	24	40	59
22	18	42	0.06084	15	36	32	57	14	20	54
23	13	31	62	10	25	12	53	05	00	50
24	09	26	41	06	14	0.05392	48	0.02695	0.05381	45
25	04	10	19	02	04	73	43	85	62	40
26	01	00	0.05999	0.1198	0.2993	54	38	76	42	35
27	0.1196	0.02989	78	94	83	36	34	67	24	31
28	92	79	57	89	73	18	29	58	05	26
29	88	68	36	85	62	0.05299	25	49	0.05287	22
30	83	58	15	81	51	79	20	39	68	17
31	79	48	0.05895	77	41	62	15	30	50	13
32	75	37	74	73	31	44	11	21	33	08
33	71	27	54	69	21	26	06	12	15	04
34	67	17	33	65	11	08	02	03	0.05198	0.1299

For samples of 0.1 ml., use 10 times the factors for samples of 1.0 ml.

The factors for volumes per cent and mM of CO in Table III are calculated by Equations 6 and 7 of Van Slyke and Neill (7) (or Equations 3 and 5 on pp. 282, 283 of Peters and Van Slyke (20)), but with values for the distribution coefficient, a', of CO between the aqueous and gaseous phases lower than values given in Table I of Van Slyke and Neill or Table 27 of Peters and Van Slyke. The values of these authors are for systems in which the aqueous phase is water, or water with insufficient solutes to

depress the solubility of CO significantly below the solubility in water. In the present analyses the combined concentrations of borate, ferricyanide, and acetate depress the solubility enough to have a slight but significant effect on the factors calculated. The solubility of CO was determined at 23° by the method of Van Slyke (21) in 10 ml. portions of a mixture of blood and reagents, in the proportions present in the chamber at the

TABLE IV

Factors by Which Mm. of P_{CO} Are Multiplied to Give Gm. of Hemoglobin per 100 Ml. of Blood

Temper- ature	Sample = 2 ml. S = 7.5 ml. a = 2.0 ml. i = 1.00	Sample = 2 ml. S = 7.5 ml. a = 0.5 ml. i = 1.00	Sample = 1 ml. S = 5.5 ml. a = 0.5 ml. i = 1.00	Sample = 0.5 ml. S = 3 ml. a = 0.5 ml. i = 1.00	Sample = 0.2 ml. S = 1.7 ml. a = 0.5 ml. i = 1.00
°C.					
15	0.09147	0.02288	0.04575	0.09129	0.2281
16	14	79	58	0.09097	73
17	0.09082	70	40	64	65
18	50	62	23	32	57
19	18	53	06	00	49
20	0.08986	45	0.04489	0.08968	40
21	54	37	73	36	33
22	20	29	57	02	25
23	0.08888	21	42	0.08870	17
24	56	13	26	38	09
25	24	06	11	06	01
26	0.08792	0.02198	0.04396	0.08776	0.2193
27	62	90	80	46	85
28	32	83	65	15	78
29	02	75	50	0.08684	70
30	0.08671	68	35	54	63
31	42	60	19	24	55
32	14	52	04	0.08594	48
33	0.08584	45	0.04290	66	40
34	54	38	75	37	33

For samples of 0.1 ml., use 10 times the factors for samples of 1.0 ml.

time the CO is extracted, and was found to be 0.71 as great as the solubility in water at the same temperature. Consequently all the values for the α' of CO in Table I of Van Slyke and Neill were multiplied by 0.71 to obtain the α' values for the present calculations. The effect of the depression of α' is to depress the factors for blood samples of 1 or 2 ml. by approximately 1 part per 1000. With 0.5 and 0.2 ml. samples the depression is less.

The factors in Table IV for gm. of hemoglobin per 100 ml. were calculated by multiplying the mM factors by 16.43/10, since Bernhart and Skeggs (22) have shown that the Fe content of human hemoglobin is 0.340 per cent, indicating that 1 gm. atom of Fe, or mole of CO, combines with 16,340 gm. of human hemoglobin.

Calculation of Inactive Hemoglobin

Inactive Hb = *total Hb* (with $Na_2S_2O_4$) minus *active Hb* (without $Na_2S_2O_4$).

Importance of Consistent Deaeration of Ferricyanide-Acetate Reagent, and of Other Factors, to Prevent Error from Variable Blanks

The accuracy of the analysis depends on keeping the amount of all gases except CO, present at the time the p_1 reading is made, at a constant, and preferably a minimal, amount, for which accurate correction can be made by blank analysis. If the ferricyanide-acetate reagent contains any air, the latter will all be extracted into the gas phase with the CO when active hemoglobin (without $Na_2S_2O_4$) is determined, and will be measured with the CO. When total Hb is determined, only the N_2 of air admitted with the ferricyanide-acetate solution appears in the final gas measured. The O_2 is removed by the $Na_2S_2O_4$ present. Although the ferricyanide is added in such great excess over the $Na_2S_2O_4$ that the latter is all oxidized, it appears that the reaction of the $Na_2S_2O_4$ of the blood mixture with O_2 in the added ferricyanide reagent is quicker than the reaction with the $K_3Fe(CN)_6$, because the O_2 practically all disappears, as is shown by the extent to which blanks for total Hb fall below those for active Hb (Table V).

The effect of air added with the ferricyanide-acetate reagent is indicated by the results in Table V for c values determined for the conditions of analysis of 1 ml. blood samples.

Table V indicates that, even when the ferricyanide reagent is deaerated and stored in a syringe sealed with deaerated oil, as described for the present methods, a slight amount of air (averaging about 1.5 c.mm.) nevertheless enters the Van Slyke-Neill chamber dissolved in this reagent. For the total Hb determination (with $Na_2S_2O_4$), the maximal variation of the blank, when the deaerated reagent was prepared as directed, was ±0.5 mm. from the mean, or about 1 part in 600 of the reading obtained in total Hb determinations on normal blood. For active Hb (without $Na_2S_2O_4$) both the blank and its variation are about twice as great.

When the deaerated reagent was stored as directed, in a syringe sealed with oil that had also been deaerated, we have never observed a significant increase in the blank during a working day. When the solution was permitted to stand in the syringe till the next day, in some cases there was no increase; in others the blank was measurably higher. The difference in protection from the air may have been due to differences in the closeness of fit of syringe piston to barrel.

In the blood analyses reported in this paper, the blanks for both active and total Hb were determined each day with ferricyanide-acetate reagent. deaerated that day.

Other sources of error from non-CO gas could be failure to extract completely the N_2 of the blood and saponin-borax mixture in the first stage of the analysis, and failure to use well deaerated NaOH solution to absorb

<center>TABLE V</center>

Value of c Correction Determined with Ferricyanide-Acetate Reagent Deaerated and Stored in Different Ways

Measures to provide deaerated ferricyanide-acetate reagent	Replicate determinations	c correction measured with gas at 0.5 ml. volume; conditions for analysis of 1 ml. blood sample	
		For active Hb determination (no $Na_2S_2O_4$)	For total Hb determination ($Na_2S_2O_4$ present)
		mm.	*mm.*
No deaeration; reagent used from open vessel at room temperature approximately saturated with air	10 on different days	18.7–26.5	12.2–19.4
Deaerated and transferred to burette under air-saturated paraffin oil; used at once	2	9.5–10.7	3.8
Deaerated and transferred to syringe (Fig. 1) containing air-saturated oil			
Used at once	2	6.9	
" next day	2	11.0–11.6	6.0
Deaerated and stored in syringe (Fig. 1) containing oil also deaerated as described for present methods; used within 8 hrs. of deaeration	20 on 10 different days	1.8– 3.4	0.6– 1.3
Average........................		2.5	1.1

CO_2 at the last stage. Both these sources are, however, easily made to approximate zero if the preliminary extraction of N_2 and the deaeration and use of the 1 N NaOH are carried out as directed.

Photometric Determination of Methemoglobin

The method of Horecker and Brackett (16) which was used is based on measurement of the decrease in optical density (wave-length 8000 A) caused by adding KCN to a borate-buffered solution of the blood, of which the pH is approximately 9.4. The change from methemoglobin to cyanhemoglobin decreases the optical density to 13 per cent that of the methemoglobin; the

KCN does not affect the optical density of any other substances than methemoglobin known to be present in blood. In order to increase the sensitivity of the method for measurement of small concentrations of methemoglobin, the procedure of Horecker and Brackett was modified by diluting 1 ml. of whole blood with saponin-borax solution to 5 ml. rather than 10 ml. Measurements of optical density were made with a Beckman quart spectrophotometer, with transmitting layers of 1 cm. of solution. The photometer was calibrated by means of blood standardized for total Hb content by the carbon monoxide method described in this paper. For calibration of the photometer portions of blood thus standardized were laked, the Hb was all converted into methemoglobin by an excess of ferricyanide, and the optical density of the ferricyanide-treated, 5-fold diluted blood was measured, before and after addition of KCN. Decrease of 1 unit in optical density corresponded to a concentration of 3.90 gm. of methemoglobin per 100 ml. in the observed solution, and to 19.5 gm. of methemoglobin per 100 ml. of the original blood.

Data from a typical determination on a normal blood are

D_1 (optical density before adding KCN) $= 0.497$
D_2 (" " after " ") $= 0.493$
$D_1 - D_2$ 0.004
Methemoglobin per 100 ml. blood $= 0.004 \times 19.5 = 0.08$ gm.

The optical density readings were estimated to 0.001 density unit on a scale divided into 0.01 unit divisions, the total density readings being, as in the above example, in the neighborhood of 0.5. Probably the error of any one reading did not exceed ± 0.002 density unit, or the sum of the errors ± 0.004 unit, which would cause an error of ± 0.08 gm. of methemoglobin per 100 ml. of blood, equivalent to about ± 0.4 per cent of the total hemoglobin. The positive result for methemoglobin in the above example therefore might be obtained in a blood that had no methemoglobin, but only in the infrequent case that maximal reading errors of the two density readings occurred in opposite directions.

<center>EXPERIMENTAL</center>

Comparison of Total Hemoglobin Determinations with Blood Samples of 1.0, 0.2, and 0.1 Ml.

In Table VI are given results of quadruplicate analyses with samples of the three different volumes. The material analyzed was a dog blood of unusually high hemoglobin content.

Comparison of Active Hemoglobin Determinations by Oxygen Capacity and Carbon Monoxide Capacity Respectively

Portions of about 15 ml. each of blood from three normal men were placed in tonometers of 250 ml. capacity and rotated at room temperature of about

23° to saturate the blood with air. Rotation was continued for an hour before analyses were begun, in order to permit approximate completion of any change from inactive to active Hb (discussed later). Then three successive samples of 1 ml. of each blood were taken at approximately 20 minute intervals and analyzed for $O_2 + CO$, the rotation of the tonometer being resumed after each sample was taken. Both O_2 and CO, mixed with CO_2 and N_2, were set free from the blood by ferricyanide, the CO_2 was absorbed with sodium hydroxide solution, and the pressure of the residual $O_2 + CO + N_2$ gas was measured with the gas at 0.5 ml. volume. From the gas thus measured the physically dissolved $O_2 + N_2$ in blood saturated with air at the observed temperature and barometric pressure was estimated from the N_2 and O_2 solubilities in blood (23, 24) (the solubilities are shown

TABLE VI

Comparison of Total Hemoglobin Measurements with Blood Samples of 1.0, 0.2, and 0.1 Ml.

All P_{CO} measurements with gas at 0.5 ml. volume.

Samples of 1 ml.			Samples of 0.2 ml.			Samples of 0.1 ml.		
P_{CO}	Temperature	Hb	P_{CO}	Temperature	Hb	P_{CO}	Temperature	Hb
mm.	*°C.*	*gm. per 100 ml.*	*mm.*	*°C.*	*gm. per 100 ml.*	*mm.*	*°C.*	*gm. per 100 ml.*
422.3	23.0	18.76	84.7	24.1	18.74	41.5	22.3	18.5
422.2	23.0	18.75	84.8	24.6	18.73	41.3	22.6	18.4
423.0	23.5	18.76	84.5	24.6	18.71	42.6	23.9	18.9
422.5	23.3	18.75	85.0	23.7	18.84	42.4	24.0	18.8

by curves in Fig. 48 of Peters and Van Slyke (20)), and were subtracted from the total $O_2 + CO + N_2$ found to obtain the $O_2 + CO$ bound by the active Hb. This procedure was used instead of determination of the O_2 alone by absorption from the extracted gases with $Na_2S_2O_4$ solution, because the latter procedure fails to include the small amounts of CO bound as HbCO that are usually found in human blood; such CO, as is shown by Roughton, Darling, and Root (5), is but slowly and incompletely displaced by O_2 during even prolonged equilibration with air at room temperature. By the procedure used, any HbCO present at the end of saturation is included in the measurement of active Hb by O_2 capacity.

After the three oxygen capacity determinations had been completed, samples were taken for determination of active Hb and total Hb by the carbon monoxide methods described in this paper.

The results (Table VII) confirm a series of O_2 and CO capacities, obtained by Van Slyke and Hiller (3) with earlier methods, in failing to show a consistent difference between CO capacity and O_2 capacity in normal human

blood. In normal blood it appears that there can be but little of any hypothetical heme derivative capable of combining with CO but not with O_2. That such heme derivatives may occur in pathological blood, however, is indicated by the extraordinary figures found by Roughton, Darling, and Root (5) in a case of idiopathic cyanosis with 15.4 volumes per cent of O_2 capacity and 22.4 volumes per cent of CO capacity (without $Na_2S_2O_4$).

TABLE VII

Comparison of Active Hemoglobin Determinations in Normal Human Blood by Oxygen Capacity and by Carbon Monoxide Capacity

Approximate time interval after blood was drawn	Determination	Subject A	Subject F	Subject P
hrs.		*gm. Hb per 100 ml.*	*gm. Hb per 100 ml.*	*gm. Hb per 100 ml.*
1.2	Active Hb by O_2	14.99	16.06	14.88
1.5	" " " "	14.98	16.24	14.91
1.8	" " " "	15.13	16.21	14.90
2.1	Active Hb by CO	15.03	16.37	15.05
2.6	Total " " "	15.27	16.50	15.27

Determinations of Inactive Hemoglobin and Methemoglobin in Normal Human Blood

Venous blood, about 15 ml., was drawn from the cubital vein, usually in the morning, without regard to time elapsed since the last meal. The blood was immediately transferred from the syringe to a 25 ml. test-tube in which a film of 15 to 20 mg. of potassium oxalate had been dried. The tube was stoppered and inverted to dissolve the oxalate, and immediately three samples of 1 ml. each were taken. Two were used by analysts H and P to start simultaneous duplicate determinations of active hemoglobin by the carbon monoxide method, and the third was used for photometric methemoglobin determination. To start the analyses with minimal delay, two Van Slyke-Neill chambers were previously prepared with saponin-borax solution in their cups, and the blood samples were transferred to the gas chambers and mixed with the saponin-borax solution within 2 to 3 minutes from the time the drawing of the blood was finished. The analysis of the sample for photometric methemoglobin determination was also started within 2 to 3 minutes.

At intervals, indicated in Table VIII, additional samples were taken for analysis from the test-tube, the latter being inverted repeatedly to assure uniform mixture of the cells before each sample was taken. Samples for duplicate determinations were always taken simultaneously, in order to

TABLE VIII

Simultaneous Determinations of Inactive Hemoglobin by CO-Gasometric Method and of Methemoglobin by Cyanide-Photometric Procedure

Blood No.	Subject	Interval between bleeding and start of analysis	Gm. Hb per 100 ml. blood						Per cent of total Hb	
			Total and active by CO			Inactive by CO		Met-Hb, photometric	Inactive by CO; from mean of H and P	Met-Hb, photometric
			Form of Hb determined	Analyst P	Analyst H	Analyst P	Analyst H			
		min.								
1	Or ♀	2	Active	13.15	13.13	0.07	0.17	0.00	0.9	0.0
		76	"	13.11	13.23	0.11	0.07	0.00	0.7	0.0
		116	"	13.11	13.23	0.11	0.07	0.00	0.7	0.0
		40	Total	13.22	13.22					
		150	"	13.21	13.27					
2	Vi ♂	2	Active	15.48	15.68	0.33	0.23	0.15	1.8	0.9
		43	"	15.57	15.76	0.24	0.15	0.24	1.3	1.5
		127	"	15.70	15.81	0.11	0.10	0.07	0.7	0.4
		80	Total	15.81	15.91					
3	DVS ♂	2	Active	14.99	15.06	0.25	0.15	0.10	1.3	0.6
		69	"	15.10	15.05	0.14	0.16	0.07	1.0	0.4
		214	"	15.12	15.25	0.12	−0.04	0.12	0.3	0.8
		246	"		15.25		−0.04			
		37	Total	15.25	15.21					
		270	"	15.22						
4	DVS ♂	3	Active	14.79		0.24		0.00	1.6	0.0
		26	"	14.90		0.13			0.9	
		204	"	14.95		0.08			0.5	
		56	Total	15.02						
		165	"	15.04						
5	Ch ♂	2	Active	16.48	16.49	0.23	0.30	0.00	1.7	0.0
		79	"	16.65	16.49	0.06	0.30	0.09	1.1	0.5
		118	"	16.64	16.59	0.07	0.20	0.00	0.8	0.0
		40	Total	16.71	16.79					
6	Ch ♂	3	Active	16.35	16.55	0.33	0.16	0.09	1.5	0.5
		37	"	16.36	16.62	0.32	0.09		1.2	
		67	Total	16.66						
		107	"	16.70	16.71					
7	Ch ♂	3	Active	15.90	15.99	0.17	0.15	0.04	1.0	0.3
		267	"	15.99	15.99	0.08	0.15		0.7	
		33	Total	16.07	16.14					

TABLE VIII—*Continued*

Blood No.	Subject	Interval between bleeding and start of analysis	Gm. Hb per 100 ml. blood						Per cent of total Hb	
			Total and active by CO			Intacive by CO		Met-Hb, photometric	Inactive by CO; from mean of H and P	Met-Hb, photometric
			Form of Hb determined	Analyst P	Analyst H	Analyst P	Analyst H			
		min.								
8	We ♂	2	Active	15.38		0.22		0.00	1.4	0.0
		43	"	15.35	15.52	0.25	0.07	0.00	1.0	0.0
		120	"	15.60	15.54	0.00	0.05	0.00	0.2	0.0
		71	Total	15.60	15.59					
9	We ♂	3	Active	14.63	14.68	0.19	0.18	0.02	1.2	0.2
		120	"	14.77	14.80	0.05	0.06		0.4	
		35	Total	14.82	14.86					
10	Em ♂	2	Active	16.46	16.54	0.16	0.15	0.18	0.9	1.1
		103	"	16.51	16.56	0.11	0.13	0.02	0.7	0.1
		246	"	16.48	16.51	0.14	0.18	0.20	0.9	1.2
		35	Total	16.62	16.73					
		70	"	16.61	16.64					
11	Sp ♀	2	Active	14.61	14.66	0.12	0.09	0.06	0.7	0.4
		118	"	14.67	14.72	0.06	0.03	0.22	0.3	1.5
		154	"	14.66	14.75	0.07	0.00	0.10	0.3	0.6
		37	Total	14.73	14.74					
		78	"	14.72	14.76					
12	Ar ♂	2	Active	15.19	15.24	0.20	0.18	0.04	1.2	0.2
		92	"	15.32	15.33	0.07	0.09	0.00	0.5	0.0
		212	"	15.31	15.32	0.08	0.10	0.00	0.5	0.0
		36	Total	15.39	15.42					
13	Fo ♂	2	Active	16.78	16.70	0.26	0.29	0.15	1.6	0.9
		40	"	16.77	16 78	0.27	0.21	0.07	1.4	0.4
		108	"	16.94	16.90	0.10	0.09	0.06	0.6	0.3
		70	Total	17.04	16.99					
14	Ph ♂	2	Active	16.03	15.98	0.04	0.15	0.06	0.6	0.4
		44	"	15.99	16.08	0.08	0.05	0.04	0.4	0.2
		120	"	16.04	16.07	0.03	0.06	0.02	0.3	0.1
		80	Total	16.05	16.13					
		300	"	16.08						
15	Na ♀	3	Active	13.19	13.25	0.08	0.19	0.00	1.0	0.0
		74	"	13.38	13.37	−0.11	0.07	0.00	−0.1	0.0
		37	Total	13.27	13.44					

TABLE VIII—*Concluded*

Blood No.	Subject	Interval between bleeding and start of analysis	Gm. Hb per 100 ml. blood						Per cent of total Hb	
			Total and active by CO			Inactive by CO		Met-Hb, photometric	Inactive by CO; from mean of H and P	Met-Hb, photometric
			Form of Hb determined	Analyst P	Analyst H	Analyst P	Analyst H			
		min.								
16	KVS ♂	3	Active	15.32	15.33	0.18	0.18	0.04	1.2	0.2
		32	Total	15.47	15.61					
		72	"		15.60					
		109	"	15.53						
17	As ♀	3	Active	13.85	13.82	0.18	0.26	0.04	1.6	0.3
		32	Total	14.01	14.06					
		72	"	14.04	14.10					
18	Do ♂	3	Active	14.75	14.81	0.22	0.27	0.10	1.6	0.6
		33	Total	14.94	15.11					
		69	"	15.00	15.05					
19	Hi ♀	3	Active	14.81		0.21		0.04	1.4	0.2
		33	"	14.83		0.19			1.3	
		148	Total	15.02						
		176	"	15.02						

TABLE IX

Constancy of Measurements of Total Hemoglobin in Blood Standing at Room Temperature after Drawing

Blood samples of 2 ml.

Time between bleeding and analysis	Total Hb found per 100 ml. blood	Time between bleeding and analysis	Total Hb found per 100 ml. blood
hrs.	*gm.*	*hrs.*	*gm.*
0	14.50	3.5	14.66
0.5	14.55	4.0	14.54
1.0	14.51	4.5	14.44
1.5	14.44	5.0	14.58
3.0	14.57	5.5	14.52

Mean total Hb.. 14.53
Standard deviation from mean
  Gm. Hb per 100 ml., ±0.066
  % of mean, ±0.45

avoid differences from the slight increase of active Hb that was usually noted in successive analyses. The total Hb, however, did not show any trend of change during the 2 to 4 hours duration of each set of observations (see also Table IX). Consequently, for a given blood, the successive inactive Hb values measured by each analyst were calculated by subtracting the successive single active Hb values from the mean total Hb value obtained by that analyst.

TABLE X

Precision of Results in Table VIII for Total, Active, and Inactive Hemoglobin Estimated by Comparison of Simultaneous Duplicate Analyses by Two Analysts

Analyses with blood samples of 1 ml.

Substance determined	No. of pairs of simultaneous duplicate analyses by analysts H and P N	Mean difference, $H-P$, between two analysts M		Standard deviation of individual results from mean difference*		Probable variation in results of either analyst†
		gm. Hb per 100 ml.	per cent of total Hb	gm. Hb per 100 ml.	per cent of total Hb	per cent of total Hb
Total Hb.................	23	+0.054	+0.35	±0.058	±0.38	±0.26
Active "	40	+0.047	+0.30	±0.086	±0.56	±0.38
Inactive Hb..............	40	−0.001	±0.00	±0.095	±0.62	±0.42

* Standard deviation calculated as $\sqrt{\Sigma(d)^2/(N-1)}$ where $d = H - P - M$, H being the value found by analyst H, P the value found by analyst P.

† Calculated as the probable error = 0.674 × standard deviation.

This procedure canceled, with regard to the inactive Hb, the effects of consistent tendency of analyst H to obtain results about 1/300 higher than analyst P for both total and active Hb (see Table X).

RESULTS

Range of Inactive Hemoglobin Content of Freshly Drawn Blood

From the results in the next to the last column in Table VIII, the range of inactive Hb in the freshly drawn blood of the nineteen subjects was from −0.1 to +1.8 per cent of the total Hb, the mean being 1.3 per cent.

Constancy of Total Hemoglobin Measurements in Successive Analyses of Bloo d Standing at Room Temperature after Drawing

In Table VIII successive determinations of total Hb performed on individual blood specimens after the latter had stood for varying intervals from the moment the blood was drawn show no trend of the results to change with the duration of the interval after drawing. A further test

of the point was made by making ten successive total Hb determinations during a period of 5.5 hours after the blood was drawn (Table IX). Blood samples of 2 ml. were used. The results indicated no change during this period in the material measured as total Hb. Similar constancy was observed by Roughton, Darling, and Root (5).

Reproducibility of Results by Carbon Monoxide Method for Total, Active, and Inactive Hemoglobin

In measurements of *total Hb* the standard deviation of ±0.45 per cent from the mean in Table IX indicates that with samples of 2 ml. half the results of a series should fall within ±0.3 per cent (0.67 × s.d.) of the mean, and that 95 per cent should fall within ±0.9 per cent, figures which are consistent with results obtained in the series of Table IX.

When samples of 1 ml. were used, the results of Tables VIII and X indicate that reproducibility was about the same as that obtained with 2 ml. samples. In Table VIII are sixteen pairs of duplicate *total Hb* determinations in which the determinations of each pair were performed on 1 ml. samples by the same analyst (ten pairs by P and six by H). In only one of the sixteen pairs does the difference between duplicates exceed 0.06 gm. per 100 ml. of blood, or 0.4 per cent of the Hb determined.

In determinations of *active Hb*, successive determinations by the same analyst could not be used as exact duplicates because of the tendency of active Hb to increase at the expense of the inactive Hb during the intervals between successive analyses. Consequently an estimate of precision has been made in Table X from the agreement obtained in duplicate simultaneous analyses, in which one analysis of each pair was carried out by a different analyst. It appears that some constant difference, such as the calibration of instruments or the manner of reading the meniscus at the 0.5 ml. mark of the Van Slyke-Neill chamber, was present between the two analysts, since the results of H for both total and active Hb averaged about 1 part in 300 higher than the results of P.

For *inactive Hb*, calculated as total Hb minus active Hb, this difference between the analysts is canceled. The figures in the last column of Table X indicate that the analytical variability of the inactive Hb is somewhat greater than that of either the total or active Hb, as might be expected from the fact that inactive Hb is calculated from the other two values.

In Vitro Change of Inactive Hemoglobin to Active Hemoglobin

From Fig. 3 and Table XI it is evident that during the first 2 hours after the blood was drawn and oxalated there was a definite trend towards a decrease in the inactive Hb. This was due to increase in the active Hb; the total Hb showed no trend of change with time (see Table IX). A

similar trend of decrease of inactive Hb *in vitro* was noted by Roughton, Darling, and Root (5).   A summary of mean values for inactive Hb determined by analyses of blood made immediately after drawing and after intervals of standing at room temperature is given in Table XI.   Comparison of the ratios in the bottom row of figures of Table XI with their standard
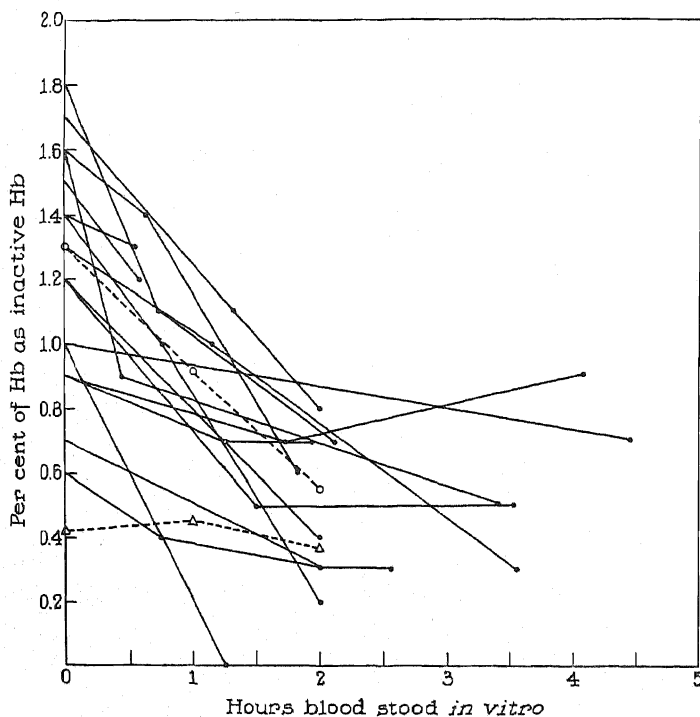
Fig. 3. Inactive hemoglobin determined in bloods that had stood for varying intervals at room temperature after drawing.   Each solid line indicates inactive Hb values obtained with one blood.   The dash line with three points marked by circles indicates average results for inactive Hb, from Table XI.   The dash line with three points marked by triangles indicates average results for photometrically determined methemoglobin, from Table XI.

deviations indicates the improbability that the observed decreases in inactive Hb with time can be due to analytical error.

Inactive Hemoglobin Other Than Methemoglobin

Methemoglobin, determined in the freshly drawn blood by the photometric KCN method, was usually lower than the inactive Hb (Fig. 3 and

Table XI); in ten of the nineteen bloods the freshly drawn blood showed only 0 to 0.3 per cent of the total Hb as methemoglobin, values within the limit of sensitivity of the method.

Unlike the inactive Hb, the methemoglobin showed no consistent trend of change during 2 to 4 hours standing after the blood was drawn.   The total amounts determined were so near the limits of measurement that the observations cannot be taken as proof that the methemoglobin content of

TABLE XI

Comparison of Results for Inactive Hb and Methemoglobin

Material determined	No. of bloods	Per cent of total Hb as inactive Hb or methemoglobin					
		Period I.Analyses begun within 3 min.		Period II. Blood stood 0.5–1.5 hrs.		Period III. Blood stood 2–4 hrs.	
		Mean	S.D. from mean	Mean	S.D. from mean	Mean	S.D. from mean
Inactive Hb*................	19	1.28	±0.35				
Methemoglobin..............	19	0.36	±0.34				
Inactive Hb*†...............	10	1.29	±0.35	0.90	±0.32	0.56	±0.23
Methemoglobin†.............	10	0.41	±0.44	0.44	±0.59	0.33	±0.44
Ratio, inactive Hb of Periods II and III to inactive Hb of Period I*†‡.............	10			0.67	±0.14	0.44	±0.20

* Each inactive Hb value used is the mean of duplicates by different analysts.

† The ten bloods in this set are those of the nineteen in the first set on which inactive Hb and methemoglobin were determined in all three periods.

‡ For methemoglobin the ratios of values of Periods II and III to Period I are not usable because about half the values are zero.

the blood did not change during the above observation periods *in vitro*, but they show little evidence of tendency towards such change.

The results indicate that freshly drawn normal human blood may contain an average of about 1 per cent of its total hemoglobin (or material measured as "total Hb" by the $Na_2S_2O_4$-CO method) in an inactive form that resembles methemoglobin in its ability to bind CO only after reduction of its Fe^{+++} to Fe^{++}, but that differs from methemoglobin in not showing the methemoglobin color reaction with cyanide, and in rapidly acquiring ability to combine with CO as the blood stands *in vitro*.   However, the sum of the errors of the determinations of methemoglobin and inactive Hb is too great to permit making this conclusion definite.

Discussion of Previous Determinations for Inactive Hemoglobin

Ammundsen (4) (see the present Table I) by Van Slyke and Hiller's (8) gasometric CO method, modified by diluting the blood with a urea solution (50 gm. per 100 ml.) instead of water, found in normal blood amounts of inactive Hb that scattered over a much wider range (up to 12 per cent of the total Hb) than those reported in the present paper.

Kallner (10, 10, *a*) also used the Van Slyke-Hiller method, with the modification that CO at about 200 mm. tension was used to saturate the blood for determination of both active Hb and total Hb, whereas Van Slyke and Hiller, and Ammundsen, used this CO tension only for total Hb, and a lower CO tension (25 to 40 mm.) for active Hb. Kallner found that when fresh venous blood, diluted with water, was shaken in the Van Slyke-Neill gas chamber with CO at 25 to 40 mm. pressure the saturation of Hb with CO was incomplete, whereas if part of the CO_2 of the freshly drawn blood was previously allowed to escape (by exposing the blood to air in an open flask, or by inverting several times in a closed flask), shaking with CO at 25 to 40 mm. accomplished maximal binding of CO. Kallner's explanation was that loss of CO_2 from the blood increased its affinity for CO, and thereby accelerated the formation of HbCO. When he shook with CO at 30 to 40 mm. tension diluted blood that had been exposed thoroughly to air, or shook freshly drawn diluted blood with CO at 200 mm. tension, constant amounts of CO were bound, which were equal, within the limits of his experimental error (*ca.* ±1 per cent), to the amounts bound after treatment with $Na_2S_2O_4$. Hence Kallner concluded that normal blood contains no methemoglobin or other inactive Hb, and that Ammundsen's positive values for inactive Hb (found in 40 per cent of her cases) were due to erroneously low active Hb values, caused by incomplete saturation of the active Hb in analyses of freshly drawn blood. We have not checked Kallner's experiments, but they offer a possible explanation for the occasional high inactive Hb values obtained by Ammundsen.

Kallner's own failure to observe definitely measurable amounts of inactive Hb may have been due partly to failure to use freshly drawn blood for the active Hb determination (see Fig. 3, present paper), and partly to the use of a technique not quite adequate for measurement of the small amounts of inactive Hb that are left after the blood has stood for some time. (In our experiments the inactive Hb 2 hours after the blood was drawn averaged only 0.4 per cent of the total Hb, equivalent to 0.08 volume per cent of CO capacity.)

The fact that our values for inactive Hb cover a lower and narrower range than those of Roughton *et al.* (5) may be due to the use of a technique in the present experiments which achieved lower blanks, and presumably lower experimental error, than the procedure of Roughton *et al.*

Method of Choice for Gasometric Hemoglobin Determination

For the most accurate blood hemoglobin values at present obtainable by gasometric analysis, and for standardizing other hemoglobin methods, the "total hemoglobin" procedure appears to be the method of choice. Results in this paper indicate that measurements of active hemoglobin, by O_2 or CO capacity, without use of reducing agents to change ferrihemoglobin to ferro-hemoglobin, will ordinarily give results about 99 per cent as high as the total hemoglobin method, and even nearer 100 per cent if the blood stands an hour or more before being analyzed.

The case of idiopathic cyanosis reported by Roughton, Darling, and Root (5), in which O_2 capacity, CO capacity, total Hb by CO $+ Na_2S_2O_4$, and photometric hemoglobin determinations as cyanhemoglobin and alkali hematin, all gave widely divergent results, indicates that hemopathological conditions, presumably rare, exist in which no method, gasometric or photometric, can be stated to give an exact measure of the hemoglobin present. It is possible that in such conditions part of the pigment material present in the cells is not identical with either the hemoglobin or the methemoglobin of normal blood; in such a case different procedures for hemoglobin determination, with factors valid for normal hemoglobin, could be expected to give different results. A survey of pathological bloods, with simultaneous photometric and gasometric total Hb values and Fe determinations, is desirable to ascertain what pathological conditions are likely to produce anomalous hemoglobin derivatives.

SUMMARY

Improved techniques for determining blood carbon monoxide and total hemoglobin, active hemoglobin, and inactive hemoglobin by the carbon monoxide capacity procedures are described.

In a series of nineteen freshly drawn normal human bloods inactive hemoglobin by the carbon monoxide method has been compared with methemoglobin determined by the cyanide reaction with the photometric procedure of Horecker and Brackett. The analyses were repeated after the blood had stood at room temperature for varying intervals up to 4 hours.

The mean methemoglobin by the photometric procedure was only 0.4 per cent of the total hemoglobin. In about half the bloods the methemoglobin percentage observed was so low (0.0 to 0.3) that the presence of methemoglobin was uncertain. The low methemoglobin values confirm Paul and Kemp. The same average value was found whether the blood was analyzed as soon as drawn or 2 or 4 hours later.

The mean inactive hemoglobin by the carbon monoxide method was 1.3 $\pm$ 0.35 per cent of the total hemoglobin when the analysis was started 2 minutes after the blood was drawn. The inactive hemoglobin decreased

as the blood stood at room temperature, and within 2 hours its average value fell nearly to the level of the photometrically determined methemoglobin.

For precise gasometric hemoglobin determinations the "total hemoglobin" method, based on measurement of CO capacity after reduction of any ferrihemoglobin present to ferrohemoglobin, is more exact than CO or O_2 capacities measured without previous reduction of ferrihemoglobin.

BIBLIOGRAPHY

1. Nicloux, M., and Fontes, G., *Bull. Soc. chim. biol.*, **6,** 728 (1924).
2. Van Slyke, D. D., *J. Biol. Chem.*, **66,** 409 (1925).
3. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, **78,** 807 (1928).
4. Ammundsen, E., *Ugesk. Laeger*, **99,** 908 (1937); *Science*, **90,** 372 (1939); *J. Biol. Chem.*, **138,** 563 (1941). Ammundsen, E., and Trier, M., *Acta med. Scand.*, **101,** 451 (1939).
5. Roughton, F. J. W., Darling, R. C., and Root, W. S., *Am. J. Physiol.*, **142,** 708 (1944).
6. Peters, R. A., *J. Physiol.*, **44,** 131 (1912).
7. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61,** 523 (1924).
8. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, **84,** 205 (1929).
9. Horvath, S. M., and Roughton, F. J. W., *J. Biol. Chem.*, **144,** 747 (1942).
10. Kallner, S., *J. Physiol.*, **104,** 6 (1945).
10, a. Kallner, S., *Acta med. Scand.*, suppl. 130, 70 (1942).
11. Conant, J. B., Scott, N. D., and Douglass, W. F., *J. Biol. Chem.*, **76,** 223 (1928).
12. Ramsay, W. N. M., *Biochem. J.*, **38,** 470 (1944).
13. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, **126,** 655 (1938).
14. Havemann, R., Jung, F., and von Issekutz, B., Jr., *Biochem. Z.*, **301,** 116 (1939).
15. Michel, H. O., and Harris, J. S., *J. Lab. and Clin. Med.*, **25,** 445 (1939–40).
16. Horecker, B. L., and Brackett, F. S., *J. Biol. Chem.*, **152,** 669 (1944).
17. Heubner, W., *Klin. Wochschr.*, **19,** 328 (1940); *Ergeb. Physiol.*, **43,** 9 (1940).
18. Paul, W. D., and Kemp, C. R., *Proc. Soc. Exp. Biol. and Med.*, **56,** 55 (1944).
19. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).
20. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1943).
21. Van Slyke, D. D., *J. Biol. Chem.*, **130,** 545 (1939).
22. Bernhart, F. W., and Skeggs, L., *J. Biol. Chem.*, **147,** 19 (1943).
23. Van Slyke, D. D., Dillon, R. T., and Margaria, R., *J. Biol. Chem.*, **105,** 571 (1934).
24. Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., *J. Biol. Chem.*, **105,** 597 (1934).

THE EFFECT OF SULFONAMIDES ON RESPIRATORY ENZYMES

By KURT I. ALTMAN*

(*From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago*)

In recent years it has been established that sulfonamides inhibit the respiration of certain bacteria, yeasts, and tissues. This inhibition was first observed on intact bacteria by Barron and Jacobs (1), and it seems to occur simultaneously with the cessation of growth in the presence of the sulfonamide (2). On the basis of numerous investigations, it can be assumed that certain relationships exist between sulfonamide inhibition of growth and of respiration. Sulfonamides may affect these processes by interfering with the synthesis of the coenzymes necessary for respiration (3). However, it seems probable that the effect of sulfonamides on bacterial respiration is only indirectly linked to their effect on growth (4–7).

The inhibitory effect of sulfonamides on respiration has not yet been completely explained, although a number of studies point in the direction in which further progress may be made. It has been shown that sulfonamides form dissociating complexes with enzymes and other proteins (8–15) and in several such cases loss of enzyme activity was found to result (16–21). The work of Dorfman, Rice, Koser, and Saunders (22) and Berkman and Koser (23) indicates that phosphopyridine nucleotides participate also in bacterial respiration and it explains the restoration of normal bacterial growth upon addition of pyridine nucleotides to the culture medium, in the presence of sulfonamides (24). It has also been reported that sulfonamides inhibit the respiration of *Plasmodium knowlesi* in the presence of glucose (25), which may be indicative of interference with oxidation of carbohydrates.

In order to establish the point at which the drug interferes with respiration, it seemed of interest to investigate the effect of sulfonamides on each of the isolated components of the respiratory enzyme system involved in the oxidation of glucose-6-phosphate. The tests applied here for the components of this system possess a high degree of specificity which permits conclusive evaluation of drug action. The results thus obtained may contribute to a more precise understanding of the inhibition caused by sulfonamides.

Effect of Sulfonamides on Cytochrome Oxidase—Cytochrome oxidase was

---

* Present address, Department of Biochemistry, University of Chicago.

prepared and tested according to the method of Haas (26). Cytochrome c was prepared according to Keilin and Hartree (27). Cytochrome oxidase activity was determined after incubating the enzyme for 30 minutes to 2 hours at 25° with 0.55×10^{-2} and 13×10^{-2} M sulfanilamide. No inhibition was observed under these conditions, thus confirming the findings of Mann and Keilin (18).

Effect of Sulfonamides on Cytochrome c—Cytochrome c was determined according to Haas (28). 2.0 mg. of cytochrome c were incubated for 30 minutes with 0.1×10^{-2} and 2.1×10^{-2} M sulfanilamide in 0.04 M phosphate buffer, pH 7.1, at 25°. Inhibition of cytochrome c oxidation was 17 and 18.5 per cent, respectively, for the two concentrations. Since the inhibition was slight and was obtained only when the drug concentration was high, this effect probably plays no rôle in the mode of action of the sulfanilamide.

Collier (29), in studying the effect of sulfanilamide on the absorption spectrum of cytochrome c, has reported the reduction of cytochrome by this drug in concentrations of 0.4×10^{-2} M.

Effect of Sulfonamides on Cytochrome c Reductase—Cytochrome c reductase was determined by measuring spectrophotometrically the rate of reduction of cytochrome c in a system and under conditions in which cytochrome reductase is the rate-determining factor (30). The components of the system were prepared as follows:

Glucose-6-phosphate was obtained by the non-enzymatic method of Levene and Raymond (31). Triphosphopyridine nucleotide was isolated from pig liver by an unpublished method of Warburg, Christian, and Griese based in principle on that used by Warburg, Christian, and Griese (32) for isolation of the nucleotide from blood corpuscles. *Zwischenferment* was prepared from Canadian top ale yeast by the method described by Haas *et al.* (30). Cytochrome c reductase was also prepared according to Haas and his coworkers (30, 33).

Cytochrome c reductase, in concentrations of 1×10^{-6} M, was incubated for 30 minutes with sulfanilamide in phosphate buffer, pH 7.5, as described in Table I. On the basis of these results, cytochrome c reductase does not appear to be the point of sulfonamide attack upon the respiratory enzyme system, and it seems more likely that the point of interference is at the dehydrogenase level. The concentration of sulfonamides required for inhibition of cytochrome reductase is considerably higher than that required for *Zwischenferment*.

Effect of Sulfonamides on Triphosphopyridine Nucleotide and Glucose-6-phosphate Dehydrogenase—The oxidation of glucose-6-phosphate to phosphohexonic acid (34) is catalyzed by an enzyme whose protein moiety, *Zwischenferment*, and whose prosthetic group, triphosphopyridine nucleo-

tide, form a dissociable complex (35). The effect of sulfonamides on this enzyme was subsequently investigated by separate incubation of the specific protein and the prosthetic group with sulfonamides at varying concentration and temperature.

Effect of Sulfonamides on Triphosphopyridine Nucleotide—1.1×10^{-5} M triphosphopyridine nucleotide was incubated at 25° with 0.7×10^{-3} and 1.4×10^{-3} M sulfanilamide in the presence of 0.02 M phosphate buffer, pH 8.3, and diluted 100 times after 30 minutes. Triphosphopyridine nucleotide activity was determined according to the micromethod of Haas *et al.* (36) by which the rate of reduction of cytochrome *c* is measured as a

<div align="center">

TABLE I

Effect of Sulfanilamide on Cytochrome c Reductase Activity
</div>

Wave-length, 550 mμ; length of absorption cell, 1.0 cm.; temperature, 25°; gas phase, air. 3.00 cc. of 0.03 M phosphate buffer, pH 7.5, + 0.03 mg. of triphospho-pyridine nucleotide + 2.8 mg. of glucose-6-phosphate + 0.13 mg. of *Zwischenferment* + 4.8 mg. of cytochrome *c*.

	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V
Sulfanilamide concentration, M			0.17×10^{-2}	1.53×10^{-2}	4.6×10^{-2}
Cytochrome *c* reductase concentration, M	4.4×10^{-9}	8.8×10^{-9}	8.8×10^{-9}	8.8×10^{-9}	8.8×10^{-9}
Rate of reduction of cytochrome *c*					
$\dfrac{\Delta \log \text{CyFe}^{+++}}{\Delta t}$, min.$^{-1}$	0.032	0.063	0.063	0.054	0.042
Inhibition, %			0	14	33.3

function of the triphosphopyridine nucleotide concentration. The results indicate that triphosphopyridine nucleotide is not affected by sulfanilamide.

Inhibition of Zwischenferment by Sulfonamides—The determination of *Zwischenferment* activity by ultraviolet spectroscopy was not found to be feasible because of absorption of sulfonamides and protein-sulfonamide complexes in the ultraviolet region. For this reason the *Zwischenferment* activity was determined by measuring the rate of decolorization of 2,6-dichlorophenol indophenol under conditions in which this rate was proportional to the *Zwischenferment* concentration (28).

It can be shown that *Zwischenferment* is strongly inhibited by sulfonamides. 0.4 mg. of *Zwischenferment* was incubated for 30 minutes with the sulfanilamide in 1.5 ml. of 0.001 M pyrophosphate buffer, pH 7.9. The enzyme solution was then diluted to 3.00 ml. and its activity tested.

Incubation beyond 30 minutes did not result in increased inhibition.   The behavior of *Zwischenferment* toward sulfonamides at varying temperatures and concentrations indicates that the drug forms a dissociable complex with this protein (Fig. 1).   The experimental evidence supporting this view is twofold.   First, incubating at lower temperature resulted in less inhibition, because at the lower temperature the concentration of free protein is diminished.   Secondly, the degree of inhibition depends upon the drug concentration because the ratio of sulfonamide to prosthetic group determines whether the enzyme protein participates in the formation
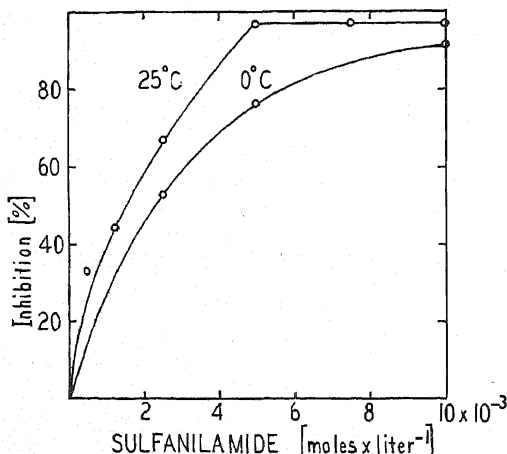

Fig. 1. Inhibition of *Zwischenferment* by sulfanilamide and effect of temperature on this inhibition.   Wave-length, 600 mμ; length of absorption cell, 1.0 cm.; gas phase, air; 3.0 cc. of 0.005 M pyrophosphate buffer, pH 8.1, + 0.01 mg. of triphosphopyridine nucleotide + 0.30 mg. of hexose monophosphate + 4.0 mg. of *Zwischenferment* + 0.055 mg. of 2,6-dichlorophenol indophenol.

of the catalytically inactive drug-protein complex or whether the active prosthetic group-protein complex is formed.

Experimentation at 38° was not possible because of the lability of *Zwischenferment;* the inhibition of *Zwischenferment* activity has been calculated by extrapolation to be approximately 50 per cent for a concentration of 1×10^{-2} M sulfanilamide.

It may be mentioned that the inhibitory effect of sulfonamides is considerably influenced by the medium during incubation; at constant sulfanilamide concentration and constant pH, the inhibition in pyrophosphate buffer was 4 times as great as in ammonium chloride-ammonium hydroxide buffer and 1.5 times as great as in phosphate buffer.

A practical application of these studies of drug action *in vitro* may be

found in the search for better chemotherapeutic agents whose effectiveness might be tested on such a system as is here employed. This point is demonstrated by comparing the inhibitory effect of a number of currently used sulfonamides. When compared on a molar basis, sulfanilamide was found to be the most effective sulfonamide in the inactivation of the protein component of glucose-6-phosphate dehydrogenase (Table II).

Antagonism between Triphosphopyridine Nucleotide and Sulfonamides— Since the formation of either the active protein-prosthetic group complex or the inactive drug-protein complex depends upon the relative proportion of drug and prosthetic group present, the effect of sulfonamides on *Zwischenferment* was considered to be competitive in nature. The results presented

TABLE II

Effect of Various Sulfonamides on Zwischenferment

Drug concentration, 0.006 M.

	Inhibition
	per cent
Sulfanilamide...	70
Sulfapyridine..	62
Sulfathiazole..	45
N¹-Benzoylsulfanilamide................................	34
Sulfadiazine...	25

in Fig. 2 indicate that inhibition of *Zwischenferment* by sulfanilamide can be prevented by simultaneous addition of prosthetic group. This can be demonstrated by incubating *Zwischenferment* with sulfanilamide and increasing amounts of triphosphopyridine nucleotide.

Thus incubation of *Zwischenferment* with 1×10^{-4} M triphosphopyridine nucleotide affords almost complete protection of the enzyme protein against the effect of 6×10^{-3} M sulfanilamide. The affinity of the enzyme protein for the prosthetic group is, therefore, about 50 times greater than its affinity for sulfanilamide. It may be assumed that other drugs which resemble triphosphopyridine more closely than sulfanilamide may displace triphosphopyridine nucleotide at even smaller concentrations.

Since the sulfonamide-protein complex is catalytically inactive as glucose-6-phosphate dehydrogenase, the respiratory pathway is blocked at this point as a result of the irreversible reaction of sulfanilamide with protein. As an illustration of the irreversibility of the drug-*Zwischenferment* reaction, it may be noted that the addition of triphosphopyridine nucleotide will not restore the activity of the enzyme once inactivation has taken place. As an indication of the specificity of triphosphopyridine

nucleotide, it may be stated that p-aminobenzoic acid, as well as split-products of triphosphopyridine nucleotide such as nicotinamide, adenylic acid, and adenosine triphosphate, is ineffective as a protective agent for the protein moiety of the enzyme.

The mechanism of action of sulfanilamide on respiration may thus be conceived of as a displacement of the coenzyme triphosphopyridine nucleotide by the sulfonamide resulting in an inactive drug-*Zwischenferment* complex. The formation of such a complex is prevented if a sufficient amount of specific prosthetic group is present while the protein is exposed to the action of the drug.
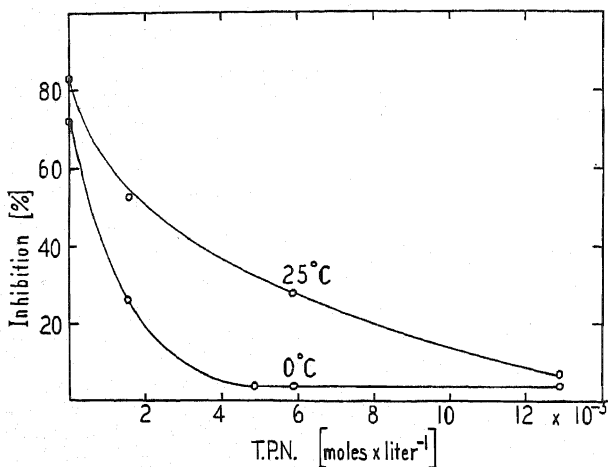

FIG. 2. Protection of *Zwischenferment* by triphosphopyridine nucleotide. Incubation of *Zwischenferment* with sulfanilamide and testing of *Zwischenferment* activity as described in Fig. 1, but various concentrations of triphosphopyridine nucleotide were added during incubation. Sulfanilamide concentration during incubation, 6.0×10^{-3} M.

Protection of the Enzyme by Its Substrate—Competition of sulfanilamide and triphosphopyridine nucleotide for the enzyme protein has been demonstrated and the mechanism of the inhibitory action of the drug was interpreted as a displacement of the triphosphopyridine nucleotide by the chemotherapeutic agent. A second mechanism for sulfonamide action is found in the displacement of the substrate, glucose-6-phosphate, from the enzyme protein by the drug. Triphosphopyridine nucleotide is 200 times as effective as glucose-6-phosphate in protecting *Zwischenferment*, which is probably due to the greater stability of the triphosphopyridine nucleotide-*Zwischenferment* complex ($K = 10^{-5}$ M) compared with the glucose-6-phosphate-protein complex ($K = 10^{-3}$ M) (28).

In the experiments presented in Fig. 3, 2 mg. of *Zwischenferment* were incubated with 5.5×10^{-3} M sulfanilamide and various amounts of glucose-6-phosphate. After 30 minutes incubation, the activity of the enzyme protein was tested as before. Antagonism between sulfonamides and metabolic substrates has been suggested on the basis of experiments with living cells (37, 38, 22), but this effect can be demonstrated more clearly with isolated enzyme systems.

Effect of Sulfanilamide on Lactic Dehydrogenase—On the basis of the experiments described in the preceding paragraphs, it seemed of interest to extend this investigation to other enzymes which require pyridine
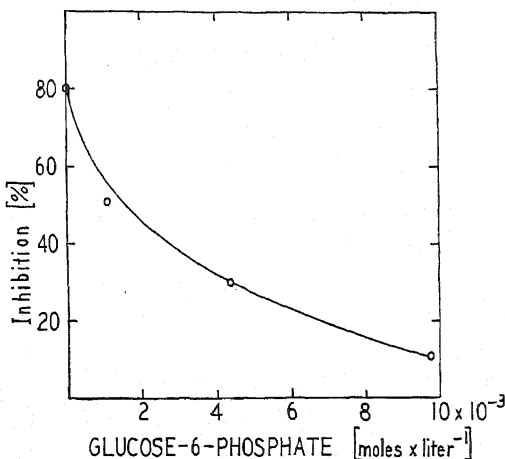

FIG. 3. Protection of *Zwischenferment* by glucose-6-phosphate. Incubation of *Zwischenferment* with sulfanilamide and testing of *Zwischenferment* activity as described in Fig. 1, except that glucose-6-phosphate in various concentrations was added during incubation. Sulfanilamide concentration during incubation, 6.0×10^{-3} M.

nucleotides as the prosthetic group. In order to test this point, lactic dehydrogenase was chosen, an enzyme containing diphosphopyridine nucleotide as the active group.

The enzyme was prepared according to Straub (39) and tested by a method recently published by Speck and Evans (40), in which 2,6-dichlorophenol indophenol is used to measure the rate of reduction of diphosphopyridine nucleotide. The results are presented in Table III.

Upon incubation of the protein moiety of lactic dehydrogenase with varying amounts of sulfanilamide for 30 minutes, a maximum inhibition of 34 per cent could be obtained. This indicates that sulfanilamide is less effective in displacing diphosphopyridine nucleotide, and that the

affinity of the drug for *Zwischenferment* is much greater than that for the protein moiety of lactic dehydrogenase.

Isolation of Zwischenferment and Triphosphopyridine Nucleotide from Escherichia coli—Assuming that one of the mechanisms by which sulfonamides interfere with bacterial respiration is the displacement of triphosphopyridine nucleotide from *Zwischenferment*, it remains to be shown that *Zwischenferment* and triphosphopyridine nucleotide are present in bacteria. The experiments of Barron and Friedemann (41) on the oxidation of hexose monophosphates suggest the presence of *Zwischenferment* in certain bacteria, but the enzyme has so far not been demonstrated in a cell-free extract.

TABLE III

Determination of Lactic Dehydrogenase and Effect of Sulfanilamide

Wave-length, 600 mμ; length of absorption cell, 1.0 cm.; temperature, 25°. 3.0 cc. of 0.02 M phosphate buffer, pH 7.2, + 0.15 mg. of diphosphopyridine nucleotide + 20 mg. of lithium lactate + 6.5 mg. of potassium cyanide + 0.055 mg. of 2,6-dichlorophenol indophenol.

	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V
Sulfanilamide concentration, M			0.15×10^{-2}	3.1×10^{-2}	5.8×10^{-2}
Lactic dehydrogenase, cc.	0.30	0.50	0.50	0.50	0.50
	Velocity of reaction (decolorization of 2,6-dichlorophenol indophenol; galvanometer deflection per 5 min.)				
	67 mm.	104 mm.	94 mm.	78 mm.	69 mm.
Inhibition, %			9.5	25	34.5

Escherichia coli was grown for 24 hours in batches of 10 liters in a medium containing glucose and Difco yeast extract. The bacteria were centrifuged down and used for isolation of the enzyme.

Zwischenferment could be isolated in two ways. (1) A bacterial paste (10 gm.) was ground in a bacterial mill according to the arrangement of Kalnitsky and coworkers (42). The bacterial juice thus obtained was extracted with 20 ml. of 0.05 M phosphate buffer, pH 7.5, and dialyzed against running distilled water at 4° for 10 hours; after centrifuging a cell-free extract was obtained. (2) A bacterial paste (approximately 15 gm.) was allowed to autolyze for 4 days at room temperature in the absence of a preservative. To the partially liquefied material 15 ml. of distilled water were added and a clear, cell-free solution was obtained after centrifugation.

Zwischenferment isolated by these two methods can be purified further by precipitation from a CO_2-saturated solution by the principle outlined by Warburg and Christian (43). Appreciable amounts of the enzyme protein could be obtained in this way and were determined by following the rate of reduction of triphosphopyridine nucleotide spectrophotometrically at 340 mμ according to the method described by Negelein and Haas (35).

In this way a minimum value for the *Zwischenferment* content of bacteria can be found which, for *Escherichia coli*, is of the order of 5.5×10^{-2} γ per

TABLE IV

Effect of Sulfonamides on Respiratory Enzyme Activity and on Oxygen Consumption of Microorganisms and Tissues

	Temperature	Sulfanilamide concentration	Inhibition	Bibliographic reference No.
	°C.	$M \times 10^2$	per cent	
Cytochrome oxidase	25	1.3	0	
" c	25	2.1	30	
" " reductase	25	4.15	30	
Triphosphopyridine nucleotide	25	1.4	0	
Zwischenferment	25	0.07	30	
	(37)*	(0.014)*	(30)*	
Escherichia coli	37	0.08	30	(46)
Staphylococcus albus	37	1.94	12	(47)
Dysentery bacillus	37	0.12	80–95	(22)
Plasmodium knowlesi	37	3.4	53	(25)
Fertilized sea urchin egg	25	4.0	45	(7)
Unfertilized sea urchin egg	25	4.0	10	(7)
Frog skin	24	0.5	40	(48)
Liver (slices)	37	2.6	19	(49)

* The figures in parentheses are extrapolated from experimental data obtained at different temperatures.

mg. of wet weight of bacteria, as based on the purest preparation of Negelein and Gerischer (44).

The ability of the enzyme protein isolated by the two procedures to function in the specific glucose-6-dehydrogenase system establishes the presence of *Zwischenferment* in *Escherichia coli*.

The presence of triphosphopyridine nucleotide has been demonstrated in bacteria by direct isolation from cell-free extracts prepared by one of the two methods described above. The extract was deproteinized with trichloroacetic acid and the mercury salt of triphosphopyridine was precipitated after neutralization with NaOH. After decomposition of the mercury salt with H_2S and treatment of the solution with barium acetate and cold

saturated barium hydroxide, triphosphopyridine nucleotide was precipitated with alcohol. The final product was tested in the specific test system described by Haas *et al.* (36) and could be substituted for triphosphopyridine nucleotide prepared from pig liver. The amount of triphosphopyridine nucleotide present in *Escherichia coli* is of the order of 3×10^{-3} γ per mg. of wet weight of bacteria, which agrees quite well with the values given for yeast (5 to 10×10^{-3} γ per mg. of fresh material) (45).

The existence of the glucose-6-phosphate dehydrogenase thus is established in *Escherichia coli*. It is presumably also present in a number of bacteria, particularly when the findings here reported are correlated with the work of Barron and Friedemann (41). It lends further weight to the feasibility of the proposed mechanism for inhibition of bacterial respiration by sulfonamides.

Table IV summarizes the effect of sulfonamides on isolated respiratory enzymes, bacteria, tissues, eggs, and parasites. Glucose-6-phosphate dehydrogenase is the respiratory enzyme we have found to be strongly inhibited by sulfonamides, and, therefore, attention is focused upon this enzyme as the possible point of interference by sulfanilamide. Since certain bacteria are known to utilize glucose-6-phosphate and have been found to contain *Zwischenferment*, it is concluded that respiration of the organism is inhibited by displacing triphosphopyridine nucleotide from *Zwischenferment*, thus interrupting the transfer of electrons from glucose-6-phosphate to cytochrome *c*.

SUMMARY

1. The effect of various sulfonamide drugs on isolated respiratory enzymes and coenzymes has been investigated. The activity of *Zwischenferment* is greatly inhibited by sulfonamides. The effect of these drugs on cytochrome *c*, cytochrome *c* reductase, and lactic dehydrogenase is much smaller, whereas triphosphopyridine nucleotide and cytochrome oxidase are not affected at all.

2. Addition of prosthetic group to the protein moiety of the dehydrogenase has been shown to protect the protein from the effect of sulfonamides, but did not reverse the sulfonamide effect. Therefore, sulfonamides react irreversibly with *Zwischenferment* and compete with the prosthetic group for the protein moiety of the enzyme.

3. The enzyme protein combines more readily with its prosthetic group than with the sulfonamide: triphosphopyridine nucleotide will counteract sulfanilamide in 50 times higher concentrations.

4. Antagonism between drug and substrate likewise has been demonstrated, since inhibition due to sulfonamides can be prevented by addition of glucose-6-phosphate.

BIBLIOGRAPHY

1. Barron, E. S. G., and Jacobs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **37**, 10 (1937).
2. Fourneau, E., and Trefouël, J., Nitti, F., and Bovet, D., *Compt. rend. Soc. biol.*, **122**, 652 (1936).
3. Dorfman, A., and Koser, S. A., *J. Infect. Dis.*, **71**, 241 (1942).
4. Sevag, M. G., and Shelbourne, M., *J. Bact.*, **43**, 421 (1942).
5. Bovarnick, M., *New York State Dept. Health, Ann. Rep. Div. Lab. and Res.*, **45**, (1943).
6. Reed, G. B., Orr, J. H., and Reed, R. W., *J. Bact.*, **48**, 233 (1944).
7. Fischer, K. C., Henry, R. J., and Low, E., *J. Pharmacol. and Exp. Therap.*, **81**, 58 (1944).
8. Klotz, I. M., *J. Am. Chem. Soc.*, **66**, 459 (1944).
9. Klotz, I. M., and Gutmann, H. R., *J. Am. Chem. Soc.*, **67**, 558 (1945).
10. Schoenholzer, G., *Klin. Wochschr.*, **19**, 790 (1940).
11. Kimmig, J., and Wesselmann, H., *Klin. Wochschr.*, **21**, 675 (1942).
12. Gilligan, D. R., *J. Pharmacol. and Exp. Therap.*, **79**, 320 (1943).
13. Fischer, S. H., Troast, L., Waterhouse, A., and Shannon, J. A., *J. Pharmacol. and Exp. Therap.*, **79**, 373 (1943).
14. Davis, B. D., *J. Clin. Invest.*, **22**, 753 (1943).
15. Reinhold, J. G., Flippin, H. F., Domm, A. H., and Pollack, L., *Am. J. Med. Sc.*, **207**, 413 (1944).
16. Zeller, E. A., *Helv. chim. acta*, **25**, 216 (1942).
17. Sevag, M. G., Shelbourne, M., and Mudd, S., *J. Gen. Physiol.*, **25**, 805 (1942).
18. Mann, T., and Keilin, D., *Nature*, **146**, 164 (1940).
19. Davenport, H. W., *J. Biol. Chem.*, **158**, 567 (1945).
20. MacLeod, C. M., *Proc. Soc. Exp. Biol. and Med.*, **41**, 215 (1939).
21. Paget, M., and Vittu, C., *Compt. rend. Soc. biol.*, **138**, 415 (1944).
22. Dorfman, A., Rice, L., Koser, S. A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, **45**, 750 (1940).
23. Berkman, S., and Koser, S. A., *J. Infect. Dis.*, **73**, 57 (1943).
24. West, R., and Coburn, A. F., *J. Exp. Med.*, **72**, 91 (1940).
25. Coggeshall, L. T., *J. Exp. Med.*, **71**, 13 (1940).
26. Haas, E., *J. Biol. Chem.*, **148**, 481 (1943).
27. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
28. Haas, E., *J. Biol. Chem.*, **155**, 321 (1944).
29. Collier, H. B., *Canad. J. Res., Sect. B*, **18**, 345 (1940).
30. Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940).
31. Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **92**, 757 (1931).
32. Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282**, 157 (1935).
33. Haas, E., Harrer, C. J., and Hogness, T. R., *J. Biol. Chem.*, **143**, 341 (1942).

34. Warburg, O., and Christian, W., *Biochem. Z.*, **242**, 206 (1931); **254**, 438 (1932).
35. Negelein, E., and Haas, E., *Biochem. Z.*, **282**, 206 (1935).
36. Haas, E., Harrer, C. J., and Hogness, T. R., *J. Biol. Chem.*, **142**, 835 (1942).
37. Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).
38. McIlwain, H., *Lancet*, **1**, 412 (1942).
39. Straub, F. B., *Biochem.*, *J.*, **34**, 483 (1940).
40. Speck, J. F., and Evans, E. A., Jr., *J. Biol. Chem.*, **159**, 71 (1945).
41. Barron, E. S. G., and Friedemann, T. E., *J. Biol. Chem.*, **137**, 593 (1941).
42. Kalnitsky, G., Utter, M. F., and Werkman, C. H., *J. Bact.*, **49**, 595 (1945).
43. Warburg, O., and Christian, W., *Biochem. Z.*, **266**, 377 (1933).
44. Negelein, E., and Gerischer, W., *Biochem. Z.*, **284**, 289 (1936).
45. Schlenk, F., in A symposium on respiratory enzymes, Madison, 105 (1942).
46. Kohn, H. I., and Harris, I. S., *J. Pharmacol. and Exp. Therap.*, **73**, 343 (1941).
47. Váczi, L., and Kiss, P. H., *Z. Immunitätsforsch. u. exp. Therap.*, **105**, 189 (1944).
48. Barnes, T. C., *J. Pharmacol. and Exp. Therap.*, **70**, 428 (1940).
49. Krems, A. D., Martin, A. M., and Dille, J. M., *J. Pharmacol. and Exp. Therap.*, **71**, 215 (1941).

THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS

III. METHIONINE

By CARL M. LYMAN, OLIVE MOSELEY, BETTY BUTLER,
SUZANNE WOOD, AND FRED HALE

(From the Texas Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station)

Simplification of the media for the microbiological determination of amino acids can be accomplished in some instances by replacing most of the amino acids with natural products. This can be done when means are available for preferentially destroying or removing one or more of the amino acids in the natural material. Thus, a medium containing acid-hydrolyzed casein has been used by Greene and Black (1) for the determination of tryptophane and the medium used by Lewis and Olcott (2) for the determination of glutamic acid contained a casein hydrolysate from which the glutamic acid had been removed by autoclaving at pH 2.9 and extracting with ethyl acetate.

The preferential oxidation of methionine with hydrogen peroxide was studied by Toennies and Callan (3). A method for removing not only methionine but also cystine, tryptophane, and tyrosine from peptone preparations by the use of hydrogen peroxide has been described by Lyman *et al.* (4). It was shown that this type of preparation was satisfactory for use in media for microbiological tests.

The present communication describes a method for the determination of methionine with *Leuconostoc mesenteroides* as the test organism and a medium in which most of the amino acid nitrogen is supplied by hydrogen peroxide-treated peptone.

One of the most useful methods for testing the reliability of assay values obtained by microbiological methods is to carry out the tests with more than one organism and, when practical, with more than one assay medium. In this investigation *Streptococcus faecalis* R was used as a second organism. Although hydrogen peroxide-treated peptone can be used in media for use with *Streptococcus faecalis* R, pure amino acids were used instead in order that the medium as well as the organism should be different in the comparative tests.

Inasmuch as the methionine values obtained by Stokes (5) and coworkers with *Streptococcus faecalis* R are somewhat lower than most of the values obtained by chemical methods, it seemed desirable to determine methionine on the same hydrolysates with a chemical method as well as

with the two microbiological methods. The colorimetric method of Mc-Carthy and Sullivan (6), as modified by Csonka and Denton (7), was chosen for this purpose.

<div align="center">EXPERIMENTAL</div>

Determination of Methionine with Leuconostoc Mesenteroides

Organism—*Leuconostoc mesenteroides* P-60 was maintained by weekly transfers as stabs in solid medium containing the following ingredients: peptonized milk 1 per cent, tryptone 1 per cent, filtered tomato juice 200 ml. per liter of medium, agar 1 per cent. Washed cells from 18 hour cultures grown on a liquid medium of the same composition as above, except for the omission of the agar, were used to inoculate the tests.

<div align="center">TABLE I</div>

<div align="center">Medium* for Determination of Methionine with Leuconostoc mesenteroides</div>

H_2O_2-treated peptone	15 gm.	Thiamine	2 mg.
Glucose	40 "	Pyridoxine	4 "
Sodium acetate	24 "	Calcium pantothenate	4 "
Ammonium chloride	12 "	Riboflavin	4 "
$l(-)$-Tryptophane	100 mg.	Nicotinic acid	4 "
dl-Tyrosine	200 "	Biotin	10 γ
$l(-)$-Cystine	200 "	Folic acid (synthetic)	3 "
Adenine sulfate	20 "	p-Aminobenzoic acid	0.2 "
Guanine	20 "	Salt Solution 1†	10 ml.
Uracil	20 "	" " 2‡	10 "
		" " 3§	10 "
		Neutralize and dilute to 1 liter	

* Medium for 200 cultures of 10 ml. final volume (5 ml. of the above medium per culture).

† Salt Solution 1, K_2HPO_4 25 gm., KH_2PO_4 25 gm., water 250 ml.

‡ Salt Solution 2, $MgSO_4 \cdot 7H_2O$ 10.0 gm., NaCl 0.5 gm., $MnSO_4 \cdot 4H_2O$ 0.5 gm., water 250 ml.

§ Salt Solution 3, $FeSO_4 \cdot 7H_2O$ 0.5 gm., water 250 ml.

Medium—The composition of the medium used for the determination of methionine with *Leuconostoc mesenteroides* is given in Table I. The glucose, ammonium chloride, and sodium acetate are added as solids and the rest of the ingredients from stock solutions preserved with a little toluene and stored in the refrigerator.

The stock solution of hydrogen peroxide-treated peptone is prepared as follows: 50 gm. of Bacto-peptone are dissolved in 250 ml. of water and 250 ml. of 2 N HCl are added after the peptone is completely dissolved. 0.025 mole of hydrogen peroxide (2.8 gm. of 30 per cent H_2O_2) is added

and the solution allowed to stand overnight at room temperature.   The material is then steamed for 30 minutes at atmospheric pressure, stirred while hot, cooled, neutralized with sodium hydroxide, and steamed again, this time for 1 hour.   The purpose of the second steaming is to decompose any hydrogen peroxide which is not used up by the oxidative reactions. The preparation is ready for use after diluting to a final volume of 1 liter. A reagent grade of hydrogen peroxide which does not contain any preservative should be used.
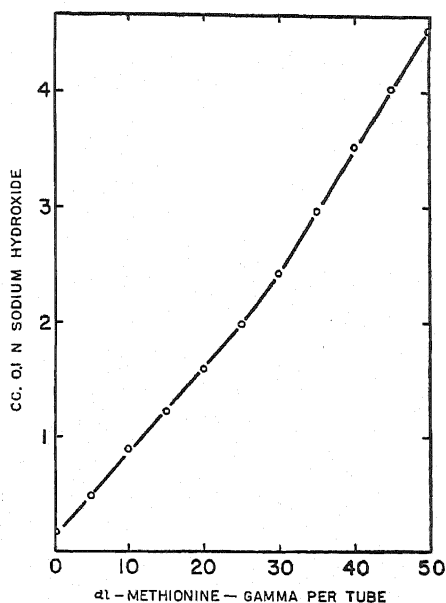

Fig. 1. Typical standard curve for the determination of methionine with *Leu-conostoc mesenteroides*.   Titration values are for 5 ml. aliquots from 10 ml. cultures.

In the preliminary phases of this investigation more hydrogen peroxide was used than is recommended here.   An improvement in the medium was obtained when the treatment of the peptone was carried out as indicated above.

Assay Procedure—Titration of the acid produced after 4 days incubation was used as a measure of the growth of the organisms.   The general procedure for carrying out the assays was the same as previously used for amino acid assays with *Lactobacillus arabinosus* (8), with the following exceptions: A constant temperature water bath at 35° was used instead of an incubator.   The period of incubation was 4 days instead of 3 days.

Standard Curve—A typical standard curve is shown in Fig. 1.   The slight

dip in the curve is not a matter of chance but always occurred with this medium.

Determination of Methionine with Streptococcus faecalis R

Medium—The composition of the medium used for the determination of methionine with *Streptococcus faecalis* R is given in Table II. In this medium a buffer consisting of a mixture of sodium succinate and sodium acetate is used instead of sodium acetate alone. *Streptococcus faecalis* is not very well adapted to growth in acid solution. Both sodium succinate

TABLE II

Medium* for Determination of Methionine with Streptococcus faecalis R

Glucose	40 gm.	dl-Alanine	400	mg.
Succinic acid	20 "	l(+)-Arginine	400	"
Sodium acetate (anhydrous)	6 "	dl-Aspartic acid	800	"
Adenine sulfate	10 mg.	l(−)-Cystine	400	"
Guanine	10 "	dl-Glutamic acid	800	"
Uracil	10 "	Glycine	400	"
Xanthine	10 "	l(−)-Histidine	400	"
Riboflavin	1 "	dl-Isoleucine	400	"
Niacin	2 "	dl-Leucine	400	"
Pyridoxamine	0.8 "	l(+)-Lysine	400	"
Thiamine chloride	0.4 "	dl-Phenylalanine	400	"
Calcium pantothenate	0.4 "	l(−)-Proline	400	"
Biotin	2 γ	dl-Serine	400	"
p-Aminobenzoic acid	2 "	dl-Threonine	400	"
Folic acid (synthetic)	10 "	l(−)-Tryptophane	200	"
Salt Solution 1	10 ml.	dl-Tyrosine	400	"
" " 2	10 "	dl-Valine	400	"
" " 3	10 "			

Add 12 gm. of NaOH pellets and finish neutralizing with NaOH solution. Dilute to 1 liter.

* Medium for 200 cultures of 10 ml. final volume (5 ml. of the above medium per culture).

and sodium citrate exert a strong buffering action at a pH range closer to neutrality than the effective range of an acetate buffer. The result of using either of these substances as buffers in media for *Streptococcus faecalis* R is to increase markedly the amount of acid formed by the organism. Sodium succinate has the advantage of producing less caramelization upon sterilization of the medium.

Assay Procedure—With a few exceptions, the method of handling the organism and of conducting the tests with *Streptococcus faecalis* R was the same as for *Leuconostoc mesenteroides*. The period of autoclaving the tubes

for sterilization was reduced to 10 minutes. The incubation time was 3 days.

Standard Curve—A typical standard curve is given in Fig. 2.

Determination of Methionine by Colorimetric Method

The method used was that of McCarthy and Sullivan (6), as modified by Csonka and Denton (7). The phospho-24-tungstic acid used for the precipitation of the basic amino acids was prepared according to the method of Wu (9).
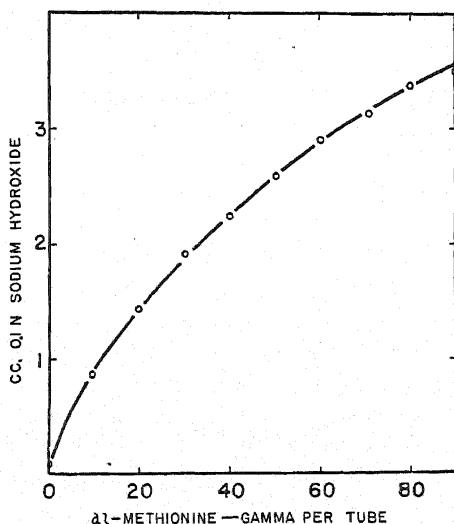

FIG. 2. Typical standard curve for the determination of methionine with *Streptococcus faecalis* R. Titration values are for 5 ml. aliquots from 10 ml. cultures.

Hydrolysis of Proteins and Foodstuffs

Except when otherwise stated, the proteins and foodstuffs were hydrolyzed by refluxing 0.5 to 2.0 gm. samples with 100 ml. of 6 N HCl for 24 hours. Most of the hydrochloric acid was removed by distillation at reduced pressure on the water bath. The hydrolysates were then neutralized and diluted to 100 ml. in a volumetric flask. If a precipitant was present, it was removed by filtration after diluting to volume.

Preparation of Egg Albumin

Egg albumin was prepared from fresh eggs. A crystalline product was obtained by the method of Kekwick and Cannan (10). The material was

then coagulated in hot water as described by Chibnall, Rees, and Williams (11).

Before the hydrogen peroxide-treated peptone was adapted for use in the *Leuconostoc* methionine medium, various modifications of a medium containing pure amino acids were tried in an attempt to improve the regularity of the standard curves and the reproducibility of the assay values. No modification of the pure amino acid medium was found which gave as consistently satisfactory results as the treated peptone medium.

Specificity tests with *Leuconostoc mesenteroides* and *Streptococcus faecalis* R showed that neither organism can use the non-natural or *d* form of methionine and that pure *dl*-methionine is a satisfactory standard since it is exactly one-half as active as *l*(−)-methionine. Homocystine and homocysteine were inactive for both organisms.

A study was made of the conditions necessary for the complete liberation of methionine from proteins by acid hydrolysis. The following three hydrolysis procedures gave methionine values which were in excellent agreement: (1) refluxing for 24 hours with 6 N hydrochloric acid; (2) refluxing for 24 hours with 6 N sulfuric acid; and (3) autoclaving at 15 pounds pressure for 6 to 8 hours with 3 N hydrochloric acid. Although autoclaving with 1 N hydrochloric acid has been successfully used for the liberation of certain other amino acids, this procedure proved to be unsatisfactory for methionine. Autoclaving for 8 hours or even longer with 1 N hydrochloric acid always gave low results.

Recovery tests with both assay organisms were carried out on a variety of different materials. In some of these tests the methionine was added to the materials before hydrolysis and in other tests the methionine was added to the neutralized hydrolysates. Satisfactory recoveries ranging from 97 to 102 per cent were obtained with both types of tests. Agreement between the values obtained at different test levels was a little more satisfactory with *Leuconostoc mesenteroides* than with *Streptococcus faecalis* R.

As a preliminary experiment in the comparison of the three methods for determining methionine, two mixtures of pure amino acids were analyzed for their methionine content. With regard to the colorimetric method, evidence was desired concerning the following points: It is known that phosphotungstic acid precipitates carry down with them some of the monoamino acids. Does the use of phosphotungstic acid for the removal of the basic amino acids, as recommended by Csonka and Denton, result in an appreciable loss of methionine? The second question is, does the glycine content of proteins and foodstuffs seriously interfere with the accuracy of this method?

Amino acid Test Mixture 1 (Table III) was patterned in a general way after the amino acid composition of casein. Since tryptophane is destroyed during acid hydrolysis, it was omitted. Amino acid Test Mixture 2 was the same as Test Mixture 1, except that the glycine was increased from 5 to 100 mg. The ratio by weight of the methionine content to the glycine content in Test Mixture 2 was therefore 1:4.

TABLE III

Amino Acid Test Mixture 1

	mg.		mg.
Alanine	55	Leucine	90
Arginine	40	Lysine	70
Aspartic acid	60	Methionine	25
Cystine	4	Phenylalanine	50
Glutamic acid	230	Proline	80
Glycine	5	Serine	60
Histidine	25	Threonine	40
Hydroxyproline	20	Tyrosine	60
Isoleucine	60	Valine	60

TABLE IV

Methionine Analyses of Amino Acid Test Mixtures

Analytical method	Test Mixture 1		Test Mixture 2	
	Methionine found	Recovery	Methionine found	Recovery
	mg.	per cent	mg.	per cent
Chemical, McCarthy and Sullivan, modified by Csonka and Denton	25.3	101.2	24.3	97.2
Microbiological, *Leuconostoc mesenteroides*	24.8	99.2	25.0	100.0
Microbiological, *Streptococcus faecalis* R.	24.6	98.4	25.2	100.8

The results of the analyses given in Table IV indicate that the use of phosphotungstic acid for the removal of the basic amino acids does not result in a significant loss of methionine and that for the purpose of evaluating foodstuffs a glycine content of 4 times that of methionine does not seriously interfere with the usefulness of the method. The figures given in Table IV are averages of values obtained at five different test levels.

Values for the methionine content of a few natural products as determined by the three different procedures are given in Table V. Possible sources of errors would be expected to be quite different in the chemical and microbiological methods. Substantial agreement between the values

obtained by the three methods was therefore considered as evidence in favor of the reliability of the methionine analyses.

In determining the methionine content of foodstuffs containing relatively large amounts of carbohydrates, one of the important problems is to make certain that the hydrolysis of the material is accomplished without undue loss of methionine as a result of humin formation or other causes. In this investigation an attempt was made to evaluate the effect of humin formation on the loss of methionine by hydrolyzing casein in the presence of various carbohydrates. The casein carbohydrate mixtures were refluxed

TABLE V

Comparison of Three Methods for Determining Methionine Effect of Presence of Carbohydrates during Hydrolysis of Protein

Material analyzed	Methionine found in protein		
	Microbiological method with		Chemical method
	Leuconostoc mesenteroides	*Streptococcus faecalis* R	
	per cent	*per cent*	*per cent*
Beef loin*.............................	2.52	2.52	2.45
" liver*.............................	2.34	2.24	2.27
Casein†.................................	2.72	2.58	2.57
" hydrolyzed in presence of equal weight of sucrose........................	2.42	2.42	2.41
Casein hydrolyzed in presence of equal weight of arabinose......................	2.49	2.55	2.46
Casein hydrolyzed in presence of equal weight of starch.........................	2.45	2.46	2.42

* Protein content calculated as nitrogen content × 6.25.
† Difco isoelectric casein; values not corrected for moisture and ash.

for 24 hours with 6 N hydrochloric acid. Methionine was then determined in the hydrolysates by three different methods.

The data given in Table V show that a small but measurable loss of methionine did take place. These data suggest that the loss of methionine, which takes place when such products as wheat and corn are hydrolyzed with strong mineral acids, is probably not large enough seriously to interfere with the usefulness of the methionine values.

Further work is needed to find a method of eliminating this loss. Hydrolysis with hydriodic acid reduces the amount of humin formation but is unsatisfactory when microbiological methods are to be used for the determination of methionine because methionine is demethylated by hydriodic acid. It is known that basic hydrolysis results in the destruction of methionine.

Table VI gives the methionine content of some proteins as determined by the use of *Leuconostoc mesenteroides* and *Streptococcus faecalis* R. The values obtained by the two organisms are in substantial agreement, although most of the values obtained with *Leuconostoc* are a few per cent higher than the values obtained with the other organism. Because of better agreement at different test levels, the values obtained with *Leuconostoc* were considered to be the more accurate.

The ash content of the casein sample was high, but the nitrogen content indicates that the sample was probably reasonably free from other impurities. The methionine value for casein obtained with *Leuconostoc*

TABLE VI

Methionine Content of Some Proteins As Determined by Two Microbiological Methods

Nitrogen and methionine values corrected for moisture and ash.

Protein analyzed	Ash content	Nitrogen content	Methionine content	
			Test organism *Leuconostoc mesenteroides*	Test organism *Streptococcus faecalis* R
	per cent	per cent	per cent	per cent
Crystalline egg albumin................	0.17	15.56	4.54	4.48
Casein, Difco isoelectric...............	1.33	15.50	2.96	2.81
Bovine plasma albumin*...............	0.53	16.14	0.81	0.77
Blood fibrin, Wilson...................	1.08	16.06	2.18	2.14
Zein†................................	0.34	15.20	1.65	1.58
Gelatin†.............................	1.06	17.84	0.88	0.83
Hemoglobin†.........................	2.28	15.65‡	1.51‡	1.53‡

* Crystalline product obtained from the Armour Laboratories.
† Commercial products.
‡ Corrected for moisture but not for ash.

mesenteroides, when calculated to a moisture- and ash-free basis, becomes 2.96 per cent. Recently, Dunn (12) and coworkers have described a microbiological method for the determination of methionine in which *Lactobacillus fermenti* is used as the test organism. The methionine content of casein reported by these workers was 3.03 per cent (corrected for moisture and ash). These values are in good agreement with the methionine content of casein as determined by a number of different chemical procedures (13–17). In general, the values obtained by the volatile iodide method (18) are somewhat higher.

Using their modification of the colorimetric method, Csonka and Denton (7) obtained a value of 2.72 (corrected for moisture and ash) for the methionine content of casein. The value of 2.57 (uncorrected) obtained by the

present authors by this same method becomes 2.80 when corrected for moisture and ash.

The methionine content of crystalline egg albumin as reported here is in good agreement with the values obtained by Lavine (17) by both the periodide titration method and the homocystine method. The figures previously reported by Kassell and Brand (14) are substantially higher. The literature values for the methionine value of crystalline egg albumin show considerably less agreement than is the case with casein. For example, Csonka and Denton (7) reported a value of 3.73 for the methionine content of their sample of egg albumin.

TABLE VII

Methionine Content of Some Foodstuffs

Material analyzed	Protein	Moisture	Methionine in foodstuffs	Methionine in protein
	per cent	*per cent*	*per cent*	*per cent*
Wheat..............................	15.36	10.94	0.197	1.28
Corn...............................	8.03	12.74	0.167	2.08
Kafir..............................	9.88	12.40	0.150	1.52
Oatmeal............................	14.76	11.44	0.236	1.60
Dried skim milk....................	33.44	6.80	0.872	2.61
Lamb chop..........................	20.91		0.512	2.45
Pork liver.........................	20.44		0.447	2.19
Peanut meal........................	38.69	7.25	0.297	0.77
Cottonseed meal....................	43.19	9.50	0.615	1.42
Soy bean meal......................	43.32	12.02	0.574	1.33
Alfalfa leaf meal..................	21.32	5.85	0.255	1.20

The methionine values obtained in the present investigation by the use of *Streptococcus faecalis* R are significantly and consistently higher than those obtained by Stokes *et al.* (5) with the same organism but a different medium.

The methionine content of some foodstuffs is given in Table VII. These data were obtained by the use of *Leuconostoc mesenteroides.*

SUMMARY

A method for determining methionine in proteins and foodstuffs by the use of *Leuconostoc mesenteroides* is described. In this method a simplified

medium is used in which most of the amino acid nitrogen is supplied by hydrogen peroxide-treated peptone.

Methionine was determined in a variety of natural products by the use of *Streptococcus faecalis* R as well as with *Leuconostoc mesenteroides*. The two microbiological methods were checked against each other and also against a colorimetric method for. determining methionine. Substantially the same values were obtained with all three methods.

Tests in which casein was hydrolyzed with acid in the presence of various carbohydrates showed that the presence of the carbohydrates during hydrolysis resulted in a small but measurable loss of methionine.

The methionine content of some proteins and foodstuffs is given.

BIBLIOGRAPHY

1. Greene, R. D., and Black, A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 322 (1943).
2. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).
3. Toennies, G., and Callan, T. P., *J. Biol. Chem.*, **129**, 481 (1939).
4. Lyman, C. M., Moseley, O., Wood, S., and Hale, F., *Arch. Biochem.*, **10**, 427 (1946).
5. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
6. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
7. Csonka, F. A., and Denton, C. A., *J. Biol. Chem.*, **163**, 329 (1946).
8. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
9. Wu, H., *J. Biol. Chem.*, **43**, 189 (1920).
10. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 277 (1936).
11. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
12. Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, **163**, 577 (1946).
13. Baernstein, H. D., *J. Biol. Chem.*, **115**, 25, 33 (1936).
14. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 145 (1938).
15. Kuhn, R., Birkofer, L., and Quackenbush, F. W., *Ber. chem. Ges.*, **72**, 407 (1939).
16. Beach, E. F., and Teague, D. M., *J. Biol. Chem.*, **142**, 277 (1942).
17. Lavine, T. F., *J. Biol. Chem.*, **151**, 281 (1943).
18. Baernstein, H. D., *J. Biol. Chem.*, **97**, 669 (1932).

THE METHIONINE CONTENT OF MEAT

By CARL M. LYMAN, BETTY BUTLER, OLIVE MOSELEY, SUZANNE WOOD, AND FRED HALE

(From the Texas Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station)

The purpose of this investigation was to obtain information on the methionine content of different kinds of meat, to determine the variability which occurs in different samples of the same kind of meat, and to compare the methionine content of corresponding organs and tissues from cattle, hogs, and sheep.

Methionine was determined in the samples by the use of a microbiological method developed in the authors' laboratory. In this method *Leuconostoc mesenteroides* is utilized as the test organism. Studies designed to test the reliability of the assay procedure have been described in a previous report (1). Substantially the same values were obtained for the methionine content of a variety of different natural products by the use of two distinct microbiological methods, the method used in the present investigation and a method in which *Streptococcus faecalis* R is utilized as the test organism.

In order to obtain meat samples which could be preserved for later investigations, the materials were dehydrated from the frozen state, extracted with anhydrous ether, and stored in desiccators in the refrigerator. The equivalence of the dehydrated samples in terms of fresh meat was known from nitrogen determinations on the freshly ground moist samples and on the dehydrated preparations. Corrections were made to take into account the small amount of nitrogen which was extracted from certain kinds of meat by anhydrous ether.

A simple but very effective apparatus for dehydrating small samples of biological materials from the frozen state is described in this report.

EXPERIMENTAL

Preparation of Samples—The different cuts of meat which consist primarily of muscle tissue were obtained at the College meat laboratory at the time the carcasses were cut up. This was usually after 4 to 7 days in cold storage. The various organs were obtained within 2 or 3 hours after the animals were killed. Some kinds of meat, such as liver, tend to lose moisture if kept in cold storage very long. The above precautions served to prevent variations in the samples due to this cause. Records of the animals

173

were kept so that it could be known with certainty that the different samples of the same kind of meat all came from different animals.

In the preparation of the samples for analyses, obvious layers of fat and large pieces of gristle were discarded. The meats were ground first with a food chopper and then with a Latapie grinder. Care was taken so that none of the juice was squeezed out and lost. Samples were taken immediately for nitrogen determinations and the balance of the material dehydrated from the frozen state. The dried material which was porous and crisp was ground in a mortar and then extracted with anhydrous ethyl
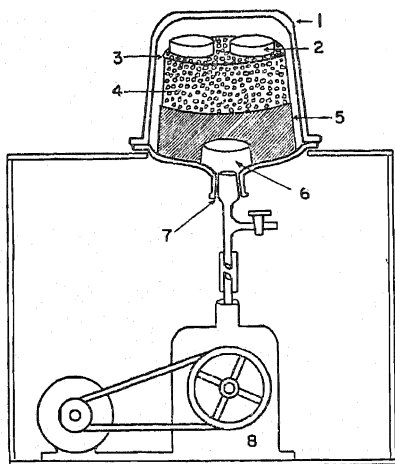
Fig. 1. Apparatus for the dehydration of small samples of ground meat or other biological materials from the frozen state: *1*, inverted Pyrex desiccator; *2*, Petri dishes containing samples; *3*, cylinder of screen wire; *4*, drierite or other granular desiccant; *5*, cylinder of light weight sheet iron; *6*, small cylinder containing cotton, covered with screen wire; *7*, standard taper Pyrex joint, No. 34/45; *8*, vacuum pump.

ether. Samples which did not powder well on the first grinding were reground after the ether extraction. Nitrogen determinations were made on the dried powder and on the extracted fat. In a few cases the nitrogen in the fat amounted to as much as 1 per cent of the total nitrogen. When this was the case, the nitrogen content of the extracted fat was taken into consideration in calculating the equivalence of the dried samples in terms of fresh meat.

The equipment used for the dehydration of the samples is shown in Fig. 1. This apparatus can be readily assembled from parts which are usually on hand in a biochemical research laboratory. The light metal cylinders, Nos. 5 and 6 on Fig. 1, are fastened to the desiccator top with adhesive tape. The desiccator bottom, which serves as a cover for the apparatus, can be easily removed for introducing or removing the samples. Because of the

short vapor path, a good vacuum is maintained in the apparatus. The evaporation is rapid enough so that samples introduced at room temperature become frozen in 5 to 10 minutes. They stay frozen until practically all of the moisture is gone.

Hydrolysis of the samples in preparation for the methionine determinations was carried out by refluxing 0.5 gm. samples with 100 ml. of 6 N hydrochloric acid for 24 hours. Most of the hydrochloric acid was distilled

TABLE I

Methionine Content of Meats

Tissue	Methionine in fresh tissue							Methionine in protein,* average values
	Sample 1		Sample 2		Sample 3		Average methionine content	
	Protein	Methionine	Protein	Methionine	Protein	Methionine		
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Beef loin...............	20.20	0.52	22.65	0.55	22.10	0.54	0.54	2.47
" brisket..............	18.85	0.48	21.28	0.52	21.32	0.52	0.51	2.47
" round...............	22.05	0.55	19.88	0.50			0.52	2.50
Pork loin................	20.14	0.49	20.67	0.51	20.78	0.50	0.50	2.44
Lamb chop..............	18.85	0.47	20.91	0.51	20.12	0.47	0.48	2.42
Beef liver..............	17.94	0.40	18.56	0.41	18.69	0.41	0.41	2.23
Pork "	19.91	0.41	20.44	0.45	18.09	0.40	0.42	2.18
Lamb "	22.25	0.47	20.13	0.39	21.00	0.43	0.43	2.07
Beef tongue.............	17.65	0.40	16.15	0.38	17.60	0.38	0.39	2.26
Pork "	16.31	0.39	16.25	0.38	15.18	0.38	0.38	2.41
Beef heart.............	17.35	0.42	18.20	0.42	17.28	0.42	0.42	2.38
Pork "	17.59	0.42	16.34	0.38	16.88	0.39	0.40	2.36
Lamb "	16.62	0.36	16.41	0.36	16.12	0.35	0.36	2.19
Beef kidney............	16.95	0.36	18.22	0.38	18.10	0.37	0.37	2.09
Pork "	15.72	0.33	15.31	0.32	15.56	0.32	0.32	2.11
Lamb "	15.94	0.32	15.56	0.30	15.54	0.30	0.31	1.99
Beef brain..............	10.75	0.22	10.65	0.21	10.55	0.21	0.21	2.10
" thymus.............	15.90	0.22	16.40	0.23	15.50	0.22	0.22	1.40
" spleen.............	18.30	0.35	18.35	0.36	18.60	0.36	0.36	1.93

* This is equivalent to calculating to 16 per cent nitrogen.

off on the water bath at reduced pressure. Water was added and the solution neutralized with sodium hydroxide. If any precipitate was present, it was removed by filtration after diluting to a definite volume. Methionine was determined on aliquots by the method of Lyman *et al.* (1).

RESULTS AND DISCUSSION

Table I shows the protein and methionine content of the fresh meat samples and also the average methionine content of the meat proteins. In general, the variation in the methionine content of different samples

of the same kind of meat was quite small.   Tender cuts of meat such as loin contained no more or no less methionine than such cuts as brisket. In fact, cuts of meat which consist mostly of muscle tissue all had very nearly the same methionine content, whether they were from beef, pork, or lamb.   It should be kept in mind that in this investigation obvious layers of fat and gristle were removed during the preparation of the samples.

The proteins of the various organs showed significant differences in their methionine content but the protein of the same organ from beef, pork, and lamb all had about the same methionine content.

Only a very limited number of values for the methionine content of different kinds of meat are to be found in the literature.   Beach, Munks, and Robinson (2) applied both their gravimetric method and the McCarthy-Sullivan (3) colorimetric method to the determination of methionine in animal tissue proteins.   The values reported by Beach and coworkers (2) are somewhat higher than the values given in Table I of the present report.

Recently, Csonka and Denton (4) have pointed out a source of error in the original colorimetric method and have proposed a modified procedure. Using the modified method, Lyman et al. (1) obtained values for the methionine content of beef liver and beef loin which are in substantial agreement with the values reported in the present communication.

SUMMARY

The methionine content of 56 samples of meat was determined by a microbiological assay procedure.   The test organism was Leuconostoc mesenteroides.

The samples of meat included beef loin, round, brisket, liver, heart, kidney, tongue, brain, thymus (sweetbreads), and spleen as well as pork loin, liver, heart, kidney, and tongue and lamb chops, liver, kidney, and heart.

Variations in the methionine content of the same kind of meat, taken from different individual animals, were very small.   Furthermore, the methionine content of a given type of tissue was very nearly the same, regardless of whether it came from beef, hog, or sheep.   Some differences were found between the methionine content of the protein material of the different organs such as liver, heart, kidney, and brain.

BIBLIOGRAPHY

1. Lyman, C. M., Moseley, O., Butler, B., Wood, S., and Hale, F., J. Biol. Chem., **166**, 161 (1946).
2. Beach, E. F., Munks, B., and Robinson, A., J. Biol. Chem., **148**, 431 (1943).
3. McCarthy, T. E., and Sullivan, M. X., J. Biol. Chem., **141**, 871 (1941).
4. Csonka, F. A., and Denton, C. A., J. Biol. Chem., **163**, 329 (1946).

THE DETERMINATION OF VITAMIN A AND CAROTENE IN SMALL QUANTITIES OF BLOOD SERUM

By OTTO A. BESSEY, OLIVER H. LOWRY, MARY JANE BROCK, AND JEANNE A. LOPEZ

(*From the Division of Nutrition and Physiology, The Public Health Research Institute of the City of New York, Inc., New York*)

For purposes of nutritional surveys and experimental studies, it was found necessary to have a method for measuring vitamin A and carotene on 0.1 ml. or less of serum in order that finger blood might be utilized or that undue amounts of blood need not be withdrawn from small experimental animals. Existing methods require at least 1 ml. of serum (1–4) and therefore necessitate venipuncture, which is time-consuming and for which consent is often difficult to obtain from subjects, particularly children, of a nutritional survey. It was also felt necessary to establish a procedure whereby large numbers of analyses could be performed without the expenditure of undue analytical time.

The usual Carr-Price (5) (antimony trichloride) reaction for vitamin A presents great difficulties when attempts are made to adapt it to small scale work. The volatility of the solvents used, petroleum ether and chloroform, makes the necessary manipulations very difficult and even slight evaporation of the chloroform results in condensation of moisture, with resultant turbidity from the antimony trichloride reagent. Furthermore, the evanescent nature of the blue color obtained renders colorimetry very difficult on a small scale. Therefore, attention was directed to the measurement of vitamin A by its absorption in the ultraviolet (328 mμ) in spite of the fact that the color intensity is only about one-third as great as with the antimony trichloride reagent. The direct ultraviolet absorption of vitamin A has been greatly limited in analytical usefulness except for measurements of high potency oils, owing to its non-specificity, since other compounds likely to be present contribute to the absorption at 328 mμ (6, 7). However, Little (8) has partially circumvented this difficulty by measuring the absorption before and after irradiation with ultraviolet light of wave-lengths 310 to 400 mμ, which destroyed chiefly vitamin A in the oils and tissues tested. Little's paper (8) gives references to those who previously made use of this principle on a limited scale and in a variety of ways for the analysis of foodstuffs. Chevallier *et al.* (9) have used an irradiation method in measurement of vitamin A in larger volumes of serum. However, the possibilities of this technique have never been fully explored, particularly in reference to blood analysis.

By utilizing a destructive irradiation technique it has been possible to develop a satisfactory method for measuring vitamin A and carotene in 60 c.mm. (0.06 ml.) of serum. Even smaller volumes of serum (35 c.mm.) can be used, if greater attention is paid to technical details. With this procedure, one analyst can measure the vitamin A and carotene in at least 50 sera in a working day.

The proposed method depends on (1) saponification and extraction of the vitamin A and carotene from serum on a micro scale with solvents of low volatility; (2) measurement of the light absorption of the small volumes at 328 and 460 mμ; (3) destruction of the vitamin A absorption at 328 mμ without affecting the absorption of other compounds at this wave-length; and (4) remeasurement of the absorption at 328 mμ.

Reagents and Apparatus—

1. 1 N KOH in 90 per cent ethyl alcohol (1 volume of 11 N KOH plus 10 volumes of absolute alcohol). The reagent should be prepared the day it is used. If color develops rapidly or if the reagent gives a blank, the alcohol should be refluxed with KOH and redistilled before use.

2. Kerosene-xylene mixture (1:1). Xylene, c.p., and odorless (water-white) reagent kerosene (obtainable from Eimer and Amend, New York).

3. Test-tubes 10 cm. × 3 mm.; 20 cm. lengths of tubing, 3.0 to 3.5 mm. internal diameter, are cleaned by boiling in half concentrated nitric acid, rinsed, dried, and divided in the middle with a hot, narrow, blast lamp flame to yield two tubes ready for use. Pyrex tubes have been used but presumably soft glass would be satisfactory and easier to seal in the flame.[1]

4. Soft glass tubes similar to those described above but only 4 cm. long and 2.5 to 3.0 mm. internal diameter. These are made and cleaned in the same fashion.[1]

5. Lang-Levy constriction pipettes, 60 c.mm. (10, 11). It is desirable to have the upper constriction quite small to permit the pipetting of the organic solvents with low surface tension. If the tip is slender and the bend in the end very short, it will facilitate the measurement of samples into the long narrow tubes.

6. General Electric B-H4 mercury discharge lamp with purple envelope and with its special transformer.

7. Arrangement for irradiating samples in the soft glass tubes (Fig. 1). When the racks are in position around the lamp, the brightest part of the light source should be opposite the lower half of the tubes so that this portion of the tubes receives full illumination. The shadow of the electrode support must not fall on any tube. A moderate air current from a fan must be used to keep the tubes cool.

---

[1] It has been found easier to make new tubes than to clean old ones, since after one end is sealed, cleaning is somewhat laborious owing to the narrow bore.

8. A Beckman spectrophotometer fitted with a micro attachment and 2 mm. quartz cuvettes (12). (The micro attachment and cells are obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

9. Racks about 5 × 5 × 2 inches to hold 100 long tubes. These may be made from wire screen (two pieces of ½ inch mesh and one piece of ⅛ inch mesh) or from sheet metal.
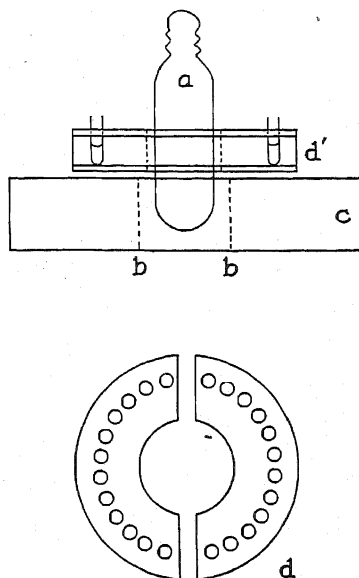

FIG. 1. Arrangement for ultraviolet irradiation. The mercury lamp (*a*) is held vertically in a clamp, base up, with the other and extending 3 or 4 cm. into a hole 8 cm. in diameter, *b-b*, in a large block of wood, *c*, which serves as a base. Semicircular racks (*d, d'*) are provided for holding the glass tubes in a circle equidistant from the lamp (6 cm. from the center of the lamp). These racks may be made from pieces of ¼ inch plywood held about 2 cm. apart, with the upper piece drilled to hold the tubes. Twenty or thirty holes may be drilled in each rack along a semicircular line.

10. The head of an eightpenny nail is cut off, and the nail is slightly flattened for a distance of 10 or 15 mm. and inserted in a small high speed hand drill (*e.g.*, Handee grinder, Chicago Wheel Manufacturing Company, Chicago) with the end projecting about 20 mm.

Procedure

Into the long slender tubes are put 60 c.mm. of serum and 60 c.mm. of alcoholic KOH. If the solutions do not run to the bottom, they are sent

down by a whipping motion. Mixing is accomplished by touching the side of the tube near the bottom to the whirling nail in the motor drill which has been mounted in a clamp with the nail up. The tube is immersed (along with others in a rack) in a water bath at 60° for 20 minutes, cooled, and 60 c.mm. of the kerosene-xylene mixture are added. Extraction is accomplished by holding the tubes at about a 45° angle against the whirling nail in such a manner that the contents are violently agitated for 10 or 15 seconds. They are then centrifuged 10 minutes at 3000 R.P.M. The tubes should be at room temperature or a little below before they are centrifuged. Each tube is cut with a file just above the kerosene-xylene layer and this layer is pipetted into the special narrow Beckman cuvette (12); all the solution possible is used, but with great care to avoid any of the aqueous layer which would cause turbidity. The pipetting is best accomplished with a fine tipped constriction pipette of about 50 or 60 c.mm. volume. However, this pipette need not be calibrated. The constriction acts merely as a brake to prevent the sample from being accidentally drawn up too far in the pipette.

Readings are made at 460 and 328 mμ. The sample is then removed to a short soft glass tube (item (4) above) and irradiated along with the other samples with the B-H4 lamp. The lamp must have been turned on at least 10 minutes prior to the beginning of the irradiation. The necessary irradiation time (30 to 60 minutes) should be determined by trial with known vitamin A solutions. Irradiation is applied for 6 or 8 times as long as is found to be necessary to destroy 50 per cent of the vitamin A in pure solutions. After irradiation, a second reading at 328 mμ is taken. In order to eliminate the danger of a turbidity of unestablished origin which some-times develops and which may be so slight as to be unnoticeable and yet serious enough to cause real error, the pipette used to transfer the sample back into the cuvette after the irradiation is rinsed before each sample with anhydrous propionic acid for one-third to one-half of its length below the constriction. This procedure neither adds significantly to the an- alytical time nor changes the volume enough to cause error. If necessary or desired, the propionic acid may be added prior to the first reading, since its incorporation does not affect the course of irradiation.[2]

Calculation—$E_{460} \times 480$ = micrograms per cent of carotene. ($E_{328} - E$ irradiated 328) $\times 637$ = micrograms per cent of vitamin A. E = optical density with a cell having a 1 cm. light path = 2 minus log per cent trans-

---

[2] A substitute for propionic acid, which may possibly be more satisfactory, is a 1:1 mixture of xylene and 2-methyl-2,4-pentanediol. The addition of a great excess of propionic acid may in some cases itself induce turbidity, whereas pentanediol ap-pears to be miscible with serum extracts in all proportions. The xylene is added to reduce viscosity.

mission with such a cell. Optical density is given directly on the Beckman spectrophotometer. If the volumes of serum and kerosene-xylene are not equal, these must be multiplied by (volume of kerosene) − (xylene)/(volume of serum).

The factor of 637 for vitamin A is based on an $E_{1\,cm.}^{1\%}$ for vitamin A palmitate in alcohol of 1720 at 328 mμ, calculated as free alcohol (13). Since vitamin A ester has only 96 per cent as much absorption in kerosene-xylene and still has 3 per cent of its initial absorption after irradiation, and since furthermore the absorption is reduced 2 per cent owing to the necessity of using a wide spectral band (8 mμ), the net $E_{1\,cm.}^{1\%} = 1720 \times 0.96 \times 0.97 \times 0.98 = 1570$. $1,000,000/1570 = 637$. The extinction coefficient of the vitamin A ester was used rather than that of the free alcohol, since most of the vitamin A in serum is esterified and saponification is quite incomplete. The factor of 480 for carotene was obtained by measuring the absorption of β-carotene (Smaco) in kerosene-xylene ($E_{1\,cm.}^{1\%} = 2080$).

If desired, the volume of serum and reagents may be increased or decreased proportionately. The ratio of serum to alcohol must be kept constant, but the amount of kerosene-xylene may be varied independently.

DISCUSSION

Saponification and extraction are conveniently carried out in the long slender tubes which prevent undue evaporation during saponification and which give sufficient fluid depth to facilitate subsequent removal of the organic solvent layer. Saponification presents no problem and it scarcely prolongs the analytical time, since it is as easy to add alcoholic KOH as it is to add alcohol alone, which must be added in any event, and as many as 100 samples may be saponified at once in a single water bath. The saponification is not complete, glycerides are only partially hydrolyzed, and the same is probably true for vitamin A esters. However, the alkaline treatment accomplishes its purpose; *viz.*, facilitation of vitamin A extraction and the removal of interfering materials.

If a motor stirrer such as is described above is not available, mixing and extracting may be accomplished by adding a 1 cm. length of 0.041 inch diameter stainless steel wire (from the Newark Wire Cloth Company, Newark, New Jersey) and shaking. Mild agitation suffices to mix the alcohol with the serum, and after adding the kerosene-xylene, the tubes are sealed at the upper end in a flame and shaken vigorously. Up to 50 tubes may be shaken together by hand (200 or 300 times). For a large series of analyses this technique is as rapid as the one given above; however, it is necessary to take great care to prevent any serum from wetting the top of the tube, which would result in charring when the tubes are sealed off and thereby jeopardize the analysis.

A major problem was to find a solvent which would completely extract vitamin A and carotene from serum and which would permit manipulation of small volumes without undue evaporation. Petroleum ether was completely unsuited owing to its volatility; toluene was an improvement; xylene was still better; and kerosene showed practically no evaporation but failed to extract vitamin A or carotene quantitatively. A 1:1 mixture of kerosene and xylene was found to have such a low volatility that evaporation could be ignored, and recovery experiments from serum demonstrated the quantitative extraction of both pigments.

Measurement of light absorption in small volumes with the Beckman spectrophotometer has been described (12). Since the samples are transferred, after the first readings, from the absorption cuvettes to small tubes for irradiation and transferred back to the cuvettes for the second readings, there is danger that so much of the sample might be lost during the manipulations as to leave an insufficient volume for the second readings. Such loss can be prevented by using slender tipped transfer pipettes and making sure that no more than a trace of liquid is left in either cuvette, irradiation tube, or transfer pipette.

Destruction of Vitamin A—When vitamin A or vitamin A ester in kerosene-xylene is irradiated with ultraviolet light, absorption at 328 mμ rapidly decreases, leaving a residual absorption of approximately 3 per cent. However, when an unsaponified serum extract is irradiated in a quartz or Pyrex tube with an unfiltered mercury vapor lamp, the absorption at 328 mμ falls and then rises higher than its initial value. Evidently while vitamin A is being destroyed, other substances are being converted into more highly absorptive materials. This phenomenon was delayed but not completely prevented by restricting the irradiation to the wave-lengths between 310 and 400 mμ, as recommended by Little (8). Little used a Corning No. 986 filter and an aqueous potassium acid phthalate solution in front of the light source to accomplish this purpose. It has been found more convenient to utilize a light source encased in a purple envelope (General Electric B-H4), which essentially cuts out wave-lengths longer than 400 mμ, and to place the samples in ordinary soda lime ("soft") glass tubes which cut off wave-lengths shorter than 310 mμ. If, in addition to filtering the light, the serum is saponified before extraction, the absorption at 328 mμ falls to a plateau which remains unchanged with further irradiation. Saponification has also been found necessary in order to effect complete extraction of vitamin A. With saponification, the light filters are perhaps unnecessary, but it has been felt desirable to retain them as a precautionary measure. However, if the soft glass used is of such a composition as to make prolonged irradiation necessary to destroy vitamin A, Pyrex tubes may be substituted.

Fig. 2 furnishes evidence that under the prescribed conditions it is only vitamin A which is destroyed by the irradiation and that new absorbing materials are not formed.   A serum extract was irradiated for 0, 9, and 60 minutes, and the absorption curves were measured between 305 and 400 mμ.   The readings at 9 and 60 minutes were then subtracted from the
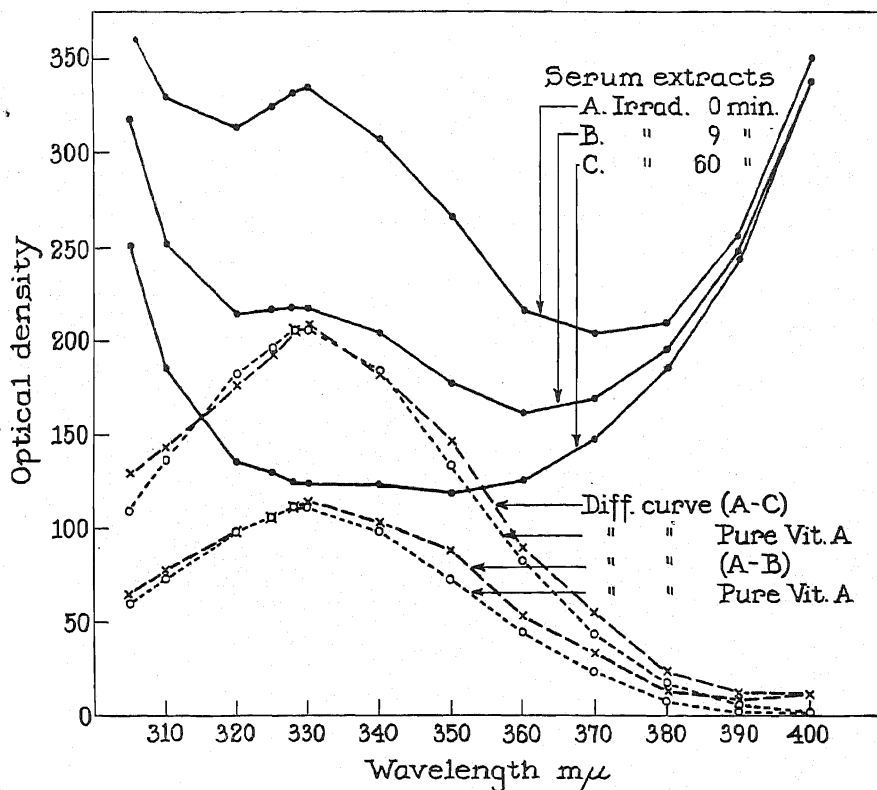

FIG. 2. Effect of irradiation on absorption of serum extracts.   Absorption curves of serum extracts before and after irradiation with ultraviolet light (310 to 420 mμ), and difference curves for serum extracts and pure vitamin A solution, obtained by subtracting the values for the absorption curves before and after irradiation.

readings at zero time to obtain difference curves representing the change in absorption induced by irradiation.   These difference curves have been compared with difference curves calculated from the change in the absorption of pure vitamin A solutions induced by prolonged irradiation.   It will be seen that the curves for pure vitamin A and serum extract difference nearly coincide.   This is strong evidence that the absorption changes are attributable to vitamin A destruction only.

Comparison with Antimony Trichloride (Carr-Price) Method—When comparisons were made between the micromethod described above and the antimony trichloride method as usually applied to serum (1–4), it was found that a number of the sera gave much higher values by the micromethod. At least part of the discrepancy was traced to certain inadequacies of the Carr-Price method as usually applied to serum. The sources of error in the antimony trichloride method proved to be (*a*) incomplete extraction of vitamin A from unsaponified serum (added vitamin A can, however, be completely extracted without saponification) and (*b*) the presence of unknown materials in some extracts which inhibited the color formation

TABLE I

Effect of Saponification on Carotene and Vitamin A Values (Antimony Trichloride Method)

The results are given in micrograms per cent.

Serum treatment	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6
Vitamin A						
A. Saponified; calculated from internal standard	45	55	62	65	69	111
B. Saponified; calculated from pure standard	42	52	57	67	65	92
C. Not saponified; calculated from pure standard	37	45	50	57	51	78
D. (C) as % of (A)	82	82	87	88	88	70
Carotene						
E. Saponified	124	232	159	171	104	176
F. Not saponified	122	189	155	166	104	169

with antimony trichloride. Comparison of the figures in Lines A and B of Table I clearly shows the importance of the use of internal standards in serum vitamin A analysis; otherwise an error is introduced owing to the inhibitory effects of materials in serum extracts on the development of the color. In Line A the vitamin A content of the serum was calculated on the basis of the increment of color produced by adding pure vitamin A to an aliquot of the serum extract immediately before color development. In Line B the vitamin A values were calculated from the color intensity produced in pure solution. A comparison of the figures in Lines B and C illustrates the effects of previous saponification on the extractability of the vitamin A from the serum.[3] It will be noted from Line D that the combined errors due to the above causes amount to from 12 to 30 per cent.

[3] The lower values in Line C are in part due to incomplete extraction and in part due to a greater inhibition of color development without saponification.

The presence of inhibitory materials in natural products which influence the rate and extent of color development resulting from the antimony trichloride reaction has long been known (14–16). Oser, Melnick, and Pader (17) have recently emphasized the value of the use of an internal standard to decrease the error resulting from this effect in analysis of oils and foodstuffs. Likewise, the importance of saponification as a means of eliminating these inhibitory materials and in aiding extraction has been previously pointed out in connection with food analysis. Analogous information in relation to serum analysis has not been satisfactory.

Yudkin (18) reported that saponification is unnecessary for the determination of vitamin A in serum, whereas others have found it to be necessary (19, 20). It is possible that the saponification employed by Yudkin resulted in the destruction of tocopherol. If this were the case, part of the vitamin A would be destroyed during the evaporation of the petroleum ether extracts and any benefits of saponification would be obscured. Pett and LePage (20) observed that vitamin A values increased with mild saponification and decreased with more drastic alkaline treatment, and in this laboratory it has been found that after prolonged saponification higher results are obtained by the Carr-Price method if tocopherol is incorporated in the petroleum ether used for extraction. With milder saponification, or none at all, the presence of tocopherol is without effect on the results. In the majority of methods in current usage for the determination of vitamin A in serum, saponification is omitted.

In order to obviate these difficulties, a modified antimony trichloride procedure was used for making comparisons with the proposed micromethod. 7 ml. of serum were saponified with 1 N KOH in 90 per cent ethyl alcohol for 20 minutes at 60°. Extracts were then made by shaking with 7 ml. of petroleum ether (b.p. 30–60°) containing 1 mg. per cent of α-tocopherol (to prevent danger of loss of vitamin A during evaporation of the extracts). Triplicate determinations were made with 1 ml. aliquots of the petroleum ether extract. The carotene was measured by absorption at 460 mμ in the Coleman model 6 spectrophotometer. The petroleum ether was then evaporated, the residue taken up in 0.1 ml. of chloroform plus 0.01 ml. of acetic anhydride, and 1 ml. of 25 per cent antimony trichloride in chloroform was added. Measurements were made at 15 seconds at 620 mμ with the same instrument. Corrections were made for the contribution of carotene to the color, assuming that all of the 460 mμ absorption was due to β-carotene. In addition, internal standards were included in triplicate by substituting 0.1 ml. of standard vitamin A solution in CHCl$_3$ for the pure CHCl$_3$. These values were utilized in computing the results. The microdeterminations were made as previously described with 60 c.mm. of serum. Eleven sera were analyzed by both the macro- (antimony trichloride) and microprocedures for vitamin A and carotene. The

averages of all the determinations were in good agreement (Table II) by the two methods. The standard deviation between individual values measured both ways was 5 γ per cent for vitamin A and 7 γ per cent for carotene (one carotene value omitted in calculating the standard deviation). This is additional proof of the reliability of the proposed micromethod for the measurement of vitamin A in serum.

Use of Different Serum Volumes and Reproducibility—Although it is recommended that 60 to 100 c.mm. of serum be used for analysis, it is possible to obtain valid data with as little as 35 c.mm. if somewhat more

TABLE II

Comparison between Micro- and Macromethods for Vitamin A and Carotene

The results are given in micrograms per cent.

Serum No.	Vitamin A		Carotene	
	Micro*	Macro† (Carr-Price)	Micro*	Macro‡
1	106	111	183	176
2	74	68	185	171
3	66	69	110	104
4	56	52	91	87
5	55	62	159	159
6	52	51	179	171
7	51	51	131	122
8	50	55	198	232
9	46	43	89	83
10	43	40	100	97
11	39	45	123	124
Average......	58	59	141	139

* Proposed method with 0.06 ml. of serum.
† Modified antimony trichloride procedure with 1 ml. of serum.
‡ Petroleum ether extract of 1 ml. of serum.

attention is given to analytical details. A number of sera were analyzed in replicate by the proposed procedure with 35, 60, and 100 c.mm. samples. The samples were treated with volumes of alcoholic KOH equal to the serum volumes and were then extracted with 40, 60, and 100 c.mm., respectively, of kerosene-xylene. Nine to twenty-eight samples were measured at each volume level. The standard deviations were 1, 1, and 2 γ per cent, respectively, for vitamin A, and 1, 1, and 1 for carotene.

Table III shows that essentially the same absolute values are obtained when different amounts of serum are used for analysis. Three sera were analyzed in triplicate at each volume level.

Effect of Storage—In making analyses for nutritional surveys it is most

convenient if samples can be collected in the field, transported to a central laboratory, and analyzed at a later date. In this case one must be assured of the keeping quality of samples and of the storage conditions compatible with the stability of the substances to be measured. To obtain the necessary information, two serum samples were stored at various temperatures in a number of sealed tubes and analyzed after 1 and after 4.5 months for vitamin A and carotene by the proposed micromethod. There was no detectable change after 1 month at either 4° or −20° in either vitamin A or carotene. At room temperature, however, the vitamin A had fallen to about 45 per cent of its initial value and the carotene to 10 per cent or less. After 4.5 months at −20° there was no significant change in vitamin A and the carotene had only fallen by about 6 per cent. At 4° the carotene

TABLE III

Vitamin A and Carotene Values with Different Volumes of Serum

	Serum 1			Serum 2			Serum 3		
Volume of serum, c.mm................	35	60	100	35	60	100	35	60	100
Vitamin A, γ %......................	74	75	74	49	47	50	50	52	50
Carotene, γ %........................	131	132	132	95	98	96	139	138	138

All measurements made in triplicate.

was almost gone in both samples, one vitamin A value was unchanged, and the other had fallen 40 per cent. It seems permissible to conclude that in serum carotene is more unstable than is vitamin A, and that sera may be stored for several weeks at 4° or several months at −20° without prejudice to the results.

SUMMARY

1. A method is described for measuring the vitamin A and carotene in 60 c.mm. of serum, an amount easily obtainable from the finger. Since, in addition, one analyst can perform at least 50 determinations in a working day, it appears to fulfil the requirements for a nutritional survey method or for studies on small animals.

2. The method has been compared with a modified Carr-Price (antimony trichloride) macroprocedure which gave essentially the same carotene and vitamin A values as the micromethod.

3. Low values for vitamin A were obtained with the Carr-Price method as usually performed on serum. Several factors which appear to be responsible for these low results are discussed.

4. Data are given on the keeping qualities of vitamin A and carotene in stored sera.

BIBLIOGRAPHY

1. McCoord, A. B., and Luce-Clausen, E. M., *J. Nutr.*, **7**, 557 (1934).
2. Kimble, M. S., *J. Lab. and Clin. Med.*, **24**, 1055 (1939).
3. May, C. D., Blackfan, K. D., McCreary, J. F., and Allen, F. H., Jr., *Am. J. Dis. Child.*, **59**, 1167 (1940).
4. Josephs, H. W., *Bull. Johns Hopkins Hosp.*, **65**, 112 (1939).
5. Carr, F. H., and Price, E. A., *Biochem. J.*, **20**, 497 (1926).
6. Morton, R. A., The application of absorption spectra to the study of vitamins, hormones and coenzymes, London, 2nd edition (1942).
7. Oser, B. L., Melnick, D., and Pader, M., *Ind. and Eng. Chem.*, *Anal. Ed.*, **15**, 717 (1943).
8. Little, R. W., *Ind. and Eng. Chem.*, *Anal. Ed.*, **16**, 288 (1944).
9. Chevallier, A., Choron, Y., and Matheron, R., *Compt. rend. Soc. biol.*, **127**, 541 (1938).
10. Levy, M., *Compt. rend. trav. Lab. Carlsberg, Série chim.*, **21**, 101 (1936).
11. Bessphs, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **164**, 321 (1946).
12. Lowry, O. H., and Bessey, O. A., *J. Biol. Chem.*, **163**, 633 (1946).
13. Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, **64**, 2407 (1942).
14. Norris, E. R., and Church, A. E., *J. Biol. Chem.*, **85**, 477 (1929–30).
15. Heilbron, I. M., Gillam, A. E., and Morton, R. A., *Biochem. J.*, **25**, 1352 (1931).
16. Corbet, R. E., Geisinger, H. H., and Holmes, H. N., *J. Biol. Chem.*, **100**, 657 (1933).
17. Oser, B. L., Melnick, D. D., and Pader, M., *Ind. and Eng. Chem.*, *Anal. Ed.*, **15**, 724 (1943).
18. Yudkin, S., *Biochem. J.*, **35**, 551 (1941).
19. Lindqvist, T., *Acta med. Scand.*, suppl. 97 (1938).
20. Pett, L. B., and LePage, G. A., *J. Biol. Chem.*, **132**, 585 (1940).

THE BIOLOGICAL SIGNIFICANCE OF THE THROMBOPLASTIC PROTEIN OF BLOOD*

By ERWIN CHARGAFF AND RANDOLPH WEST

(From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, and the Medical Service of the Presbyterian Hospital, New York)

The activators of blood clotting present in mammalian tissue, *i.e.* the agents responsible for the conversion of prothrombin to thrombin, have been shown to be lipoproteins of a very high particle weight (1–3). These substances readily form sediments in a strong centrifugal field (31,000g), but remain in solution when subjected to weaker centrifugal forces (5000g).

The consideration of the manner in which thromboplastic substances occur in blood is of importance for an understanding not only of the physiology of normal blood coagulation but also of bleeding disturbances, such as hemophilia. The rôle of the blood platelets, usually regarded as the main source of thromboplastic material, in the clotting of normal and of hemophiliac blood has often been considered. (Compare the surveys in (4, 5).) But whether an additional factor, exhibiting the centrifugal characteristics of the thromboplastic protein of tissue cells, is present in blood has remained unknown. A brief report on the effect of high speed centrifugation of plasma on its coagulation time (6) appeared, however, suggestive of the existence of such a factor.

The orienting studies presented here include a comparative investigation of the effect of high speed centrifugation on the clotting time of normal and of hemophiliac plasma.[1] They were prompted by the opportunity of studying an interesting case of an acquired hemophilia-like condition in a female patient.

The literature contains only few reports on an acquired bleeding disturbance in the female exhibiting, in a more or less typical fashion, the characteristics of hemophilia, except for the important feature of hereditary transmittal (8, 9). The case which furnished the opportunity for the study of the blood clotting defect presented here was, in addition, characterized by the presence of a circulating anticoagulant, a phenomenon occasionally observed in the past (10, 11). The other blood specimens used in this study were obtained from a genuine hemophiliac with authentic family history.

[1] The effects of centrifugation at low speed have been compared by Quick (7).

Origin of Blood Specimens—The blood samples designated N were collected from normal young adults. Those marked H came from an authentic hemophiliac. The blood specimens listed as F were obtained from a female patient with an acquired bleeding disturbance. A brief history of this case is given in the following paragraph. All blood samples were collected before breakfast and examined immediately.

Case History—(Presbyterian Hospital, Unit No. 784026.)   A housewife of 33 years was admitted with diffuse, recent subcutaneous hemorrhages and a large hemorrhage beneath the tongue. There were slight limitation of motion of both knees, secondary anemia, moderate leucocytosis. Blood clotting time, 80 to 100 minutes. Considerable prolongation of clotting time of normal blood on admixture of small amounts of the patient's blood. Prothrombin time, 23 seconds. Platelets, 165,000. Serum protein 6.1 gm. per 100 cc. Alkaline phosphatase, 2.4 Bodansky units. Cephalin flocculation, negative. Electrophoretic pattern of plasma normal, including fibrinogen. No unusual capillary fragility. Blood is Rh-positive. The present illness probably dates from 7 months before admission when the patient had her last delivery. Several weeks after a normal delivery, hemorrhages, chiefly in the arms and legs, appeared about every 2 weeks. There was subcutaneous bleeding, intramuscular and within joints. Previous bleeding history: hemorrhages following spontaneous abortion in 1942 and after a tooth extraction in 1943. Three out of five pregnancies resulted in miscarriage; neither of the living children has any bleeding tendency.

Protamine Titration—In view of the presence of a circulating coagulant in blood F it appeared of interest to examine it for the presence of heparin. The determination of the clotting time of blood and plasma, following the addition of varying small amounts of salmine, is based on the observation that this strongly basic protamine abolishes the anticoagulant action of heparin both in the circulation and *in vitro* (12). The determinations on whole blood were carried out with venous blood, freshly drawn (without any addition) before breakfast. The plasma was obtained 50 minutes later from the same blood sample without centrifugation by allowing the blood cells to settle in the refrigerator. The results, summarized in Table I, furnish no indication of the presence of an anticoagulant of the heparin type. The drop in clotting time, observed on addition of 2.5 γ of protamine, cannot be explained at present.

Effect of Centrifugation on Plasma Clotting Time—The effect of differential centrifugation on the clotting time of normal human plasma has been discussed in a recent publication (13). In the present study a similar approach was employed for a comparison of the behavior of plasma obtained from the patients F and H with that of normal plasma. The blood, collected before breakfast, was mixed immediately with one-ninth its volume of 0.1 M sodium oxalate solution. Plasma samples were removed following centrifugation at 1500 R.P.M. (260g) for 3 minutes (Experiment 1, Table II). The remain-

ing mixture was then centrifuged at 4000 R.P.M. (1900g) for 20 minutes in a refrigerated angle centrifuge. The clear supernatant was siphoned off carefully, and samples were removed for testing (Experiment 2, Table II). 10 cc. portions of the plasma samples were then subjected to a centrifugation at 20,000 R.P.M. (31,000g) for 150 minutes in a refrigerated International centrifuge equipped with a multispeed attachment,[2] which brought about

TABLE I

Effect of Protamine on Clotting Time of Blood and Plasma from Patient F

The experiments were carried out at pH 7 and 30° by mixing 0.2 cc. of blood or plasma with 0.2 cc. of the solution of salmine in physiological saline.

Clotting time	Salmine in experiment				
	25 γ	10 γ	5 γ	2.5 γ	0 γ
	min.	*min.*	*min.*	*min.*	*min.*
Blood.............	35	40	35	22	48
Plasma............	40	40	40	30	35

TABLE II

Effect of Centrifugation on Plasma Clotting Time

The experiments were performed at 37° by mixing 0.1 cc. of plasma with 0.2 cc. of a 0.01 M calcium nitrate solution (containing 0.42 per cent of sodium chloride). In experiments in which more than one clotting time is reported, the figures record the span between the first appearance of fibers and the formation of a clot. N = normal; F = female bleeder; H = hemophiliac.

Experiment No.	Duration of centrifugation	Centrifugal force	Plasma clotting time		
			N	F	H
	min.	g			
1	3	260	2 min., 50 sec.	9 min., 45 sec. to 22 min.	5 min., 10 sec. to 15 min.
2	20	1,900	3 min., 20 sec. to 4 min., 30 sec.	15–27 min.	10 min., 40 sec. to 17 min.
3	150	31,000	5 min., 50 sec. to 8 min.	No clot within 81 min.	12 min., 35 sec. to 25 min.

the sedimentation of minute reddish brown translucent pellets. The coagulability of the plasma samples following the high speed centrifugation was likewise examined (Experiment 3, Table II).

The small pellets sedimented by the centrifugation of 10 cc. of plasma at 31,000g were suspended in 1 cc. of physiological saline (corresponding to a 10-fold concentration with respect to the original plasma). The clotting

[2] All contrifugations were carried out in lusteroid tubes.

effect of these suspensions on the plasma samples that had undergone centrifugation at 31,000g is presented in Table III.

It appeared of interest to determine whether a fraction possessing the centrifugal characteristics of the thromboplastic protein was contained in the pellets sedimented from normal plasma at 31,000g. As has repeatedly been shown in this laboratory (2, 3, 13), the thromboplastic protein of vari-

TABLE III

Effect of High Speed Sediments on Clotting Time of Plasma Centrifuged at High Speed

The experiments were carried out at 37° by mixing 0.1 cc. of plasma with 0.1 cc. of the saline suspensions of the high speed sediments (or, in control experiments, of physiological saline) and 0.2 cc. of a 0.01 M calcium nitrate solution (containing 0.42 per cent of sodium chloride). The components of the mixtures are indicated by plus signs. For the explanation of the clotting intervals indicated, compare Table II. Assays A and B were carried out at different times of admission to the hospital of patient F and with different specimens of normal plasma. N = normal; F = female bleeder; H = hemophiliac.

Experiment No.	N		F		H		Clotting time	
	Plasma	Sediment	Plasma	Sediment	Plasma	Sediment	Assay A	Assay B
							sec.	*sec.*
1	+						365–690	350–480
2	+	+					100	90
3			+				No clot within 90 min.	No clot within 81 min.
4			+	+			345–405	430–580
5		+	+				170	325–380
6	+			+			305–437	354–435
7					+			755–1030
8					+	+		325–395
9		+			+			104
10	+					+		300–380
11			+			+		440–780
12			+			+		540–780

ous tissues is sedimentable at 31,000g, but not at 5000g. A sample of normal oxalated plasma was subjected to a fractional centrifugation, as described before. Following centrifugation at 1900g for 20 minutes, the clotting time of the recalcified plasma was 217 to 250 seconds; following centrifugation at 31,000g for 150 minutes, 340 to 690 seconds (see also Table II). The suspension of the high speed sediment (from 10 cc. of plasma) in 1 cc. of borate buffer of pH 8.4 was centrifuged at 8000 R.P.M. (5000g) for 30 minutes. The almost clear supernatant, when tested in the arrangement presented in Table III, produced a firm clot in 160 seconds. The amount of

thromboplastic protein found in normal human plasma, when expressed in terms of the most active preparation isolated from human tissues (3), may be estimated tentatively as between 0.1 and 1 γ per cc. of plasma.

Anticoagulant Effect of Plasma—Admixture of plasma F to normal plasma (corresponding to the plasma samples listed as Experiment 2, Table II) caused definite clotting inhibition. With 7 parts of the patient's plasma and 3 parts of normal plasma or with equal parts of both plasma samples, an extremely slow fibrin deposition was observed which led to the formation of a very soft coagulum in about 29 minutes.

TABLE IV

Effect of Thromboplastic Protein of Beef Lung on Clotting Time of Plasma Centrifuged at High Speed

The experiments were carried out at 37° by mixing 0.1 cc. of plasma with 0.1 cc. of physiological saline containing the indicated amounts of thromboplastic protein and 0.2 cc. of a 0.01 M calcium nitrate solution (containing 0.42 per cent of NaCl). For the explanation of N, F, H, A, and B, see Table III.

Experiment No.	Thromboplas-tic protein	Clotting time				
		N		F		H
		Assay A	Assay B	Assay A	Assay B	Assay B
	γ	sec.	sec.	sec.	sec.	sec.
1	100	30	33	30	43	37
2	10	41	46	42	59	60
3	1	75	82	83	109	104
4	0.1	149	160	191	230	231
5	0.01	248	265	355–945	420–559	400–560
6	0.001	295–540				
7	0	365–690	350–480	>5400	>4860	755–1030

Plasma Antithrombin—The plasma of patient F appeared to contain no more than the normal amount of antithrombin. (For a discussion of this factor, see a recent review article (14).) Albumin fractions prepared by techniques described previously (15) exhibited an antithrombin effect that was only slightly higher than that shown by comparable preparations from normal plasma.

Effect of Thromboplastic Protein—The action of preparations of the thromboplastic protein on human plasma, centrifuged at 31,000g, has recently been discussed (3, 13). In the experiments here presented a highly purified preparation of the thromboplastic protein of beef lung (2) was employed. The results, shown in Table IV, demonstrate that with less than 1 γ of the thromboplastic protein per 0.1 cc. of plasma the coagulation times of both pathological plasma samples were abnormally long, though with more this

difference vanished.   In what may be considered a region of thromboplastin excess (1 γ or more) the hemophiliac plasma H and the hemophilia-like plasma F behaved essentially similar to normal plasma.

Incubation of Thromboplastic Protein with Plasma—In view of the apparent deficiency of the pathological plasma specimens in thromboplastic protein, it was of importance to determine whether these plasma samples contained an agent able to destroy the thromboplastic protein.   Oxalated plasma (prepared by centrifugation at 31,000g for 150 minutes) was incu-

TABLE V

Effect of Incubation of Thromboplastic Protein with Plasma

Mixtures of 1 cc. of plasma (centrifuged at high speed) with 1 cc. of saline containing the indicated amounts of thromboplastic protein were kept at 37°. The clotting times were determined at stated intervals on 0.2cc. portions of the mixtures, following recalcification as in Table II. The first appearance of fibers is recorded as the clotting time.   N = normal; F = female bleeder; H = hemophiliac.

Thrombo-plastic protein per 1 cc. plasma	N		F		H	
	Incubation time	Clotting time	Incubation time	Clotting time	Incubation time	Clotting time
γ	*min.*	*sec.*	*min.*	*sec.*	*min.*	*sec.*
20	0	85	0	91	0	105
	10	105	17	115	14	107
	25	102	31	128	28	110
	40	115	48	140	44	130
	60	118	67	140	63	132
	110	132	118	155	113	190
	150	180	161	202	155	232
	240	268	258	245	250	296
1	0	150	0	215	0	205
	20	170	35	280	26	289
	77	255	92	350	85	355
	120	285	135	420	126	440

bated at 37° with a very potent preparation of the thromboplastic protein of beef lung (in concentrations of 20 and 1 γ per cc. of plasma) and the clotting time of the recalcified mixture determined after various time intervals. No important departure from the behavior of normal plasma was observed: the rates at which the clotting times of all plasma specimens responded to the length of incubation were essentially similar (Table V). It is, in fact, doubtful whether the gradual prolongation of the clotting time may be attributed to the thromboplastic protein which, at least in the absence of plasma proteins, is known to be extremely stable (2).   It is pos-

sible that partial inactivation of prothrombin is responsible for the observed effect.

DISCUSSION

Tables II to IV may be considered to summarize the most important results of this study. When oxalated plasma, following centrifugation at 4000 R.P.M. (1900g), is subjected to prolonged centrifugation at 20,000 R.P.M. (31,000g), its coagulation time is extended very noticeably. This effect is even more conspicuous in hemophiliac plasmas in which occasionally complete absence of clotting is observed (Table II). The clotting factor of which the plasma is deprived by high speed centrifugation is found in a minute pellet whose sedimentation is brought about by this operation. When the effects of these sediments on normal and pathological plasma specimens are compared, it can be seen that the plasma from patients exhibiting a marked bleeding tendency contains considerably less of the clotting activator than does normal plasma (Table III). Thromboplastic protein preparations, when added in sufficient, though still very small, amounts, induce the pathological plasma specimens to clot with normal speed (Table IV).

The studies of the coagulation defect in the case exhibiting an acquired bleeding tendency resembling hemophilia (patient F) indicate that it is attributable to two causes, (1) a pronounced lack of thromboplastic factor in the plasma of the patient, (2) the presence in this plasma of a clotting inhibitor different from heparin. It might be argued that these two causes are in reality facets of the same phenomenon; namely, the occurrence in the patient of an agent that destroys the activity of the thromboplastic protein. However, the experiments on the effect of incubation of the thromboplastic protein with plasma (Table V) furnish no support to this assumption, at least with respect to the presence in the blood of such a factor, nor is there evidence of the existence of such an agent in the authentic hemophiliac plasma H.

It appears likely that a thromboplastic protein not unlike that isolated from tissue cells occurs in extravasated normal blood and contributes to its clotting properties. The particulate fraction sedimented at 31,000g probably includes, in addition to the thromboplastic agent, a variety of minute breakdown products of the blood corpuscles. Whether the thromboplastic protein exists in *circulating* blood remains, unfortunately, an inherently unanswerable question. In any event, the results summarized in Table III point to a remarkable parallelism between the clotting behavior of the whole plasma and the activity of the high speed sediment derived from it. If the activation experiments presented in Table IV are plotted as previ-

ously described (13), it can, for instance, be computed from the results of Experiments 2 and 6 in Table III that the high speed sediment from 1 cc. of normal plasma exhibited an activity corresponding to 0.4 γ of the thromboplastic protein from beef lung, whereas the sediment from the pathological plasma F contained only 0.03 γ.

The reasons for this abnormality are not clear. Recent work in this laboratory (3, 13) has demonstrated that practically the entire thromboplastic activity of tissue cells is confined to one particulate fraction, the thromboplastic protein, which is probably derived from the cytoplasm. The specific cell types responsible for the thromboplastic effect are not yet known. In the absence of additional information, the explanation of bleeding disturbances that are due to the low concentration of the thromboplastic protein in blood (hypothromboplastinemia) will have to be limited to the statement that these conditions could be due either to the failure of certain tissue cells to produce, or to release, sufficient quantities of the thromboplastic factor or to the occurrence in the organism of an agent that destroys this factor. Concerning the anticoagulant whose presence has been shown to be probable in plasma F, all that can be said at present is that it appears not to be heparin.

SUMMARY

The effects of high speed centrifugation on the clotting behavior of normal and hemophiliac plasma are compared. The coagulation defect in the pathological specimens is shown to be attributable to a marked deficiency of the blood in a clotting factor similar to the thromboplastic protein of tissue cells. The blood of a female patient exhibiting a non-hereditary bleeding disturbance was, in addition, characterized by the presence of a coagulation inhibitor different from heparin. In connection with the finding that the coagulation defect can be overcome by the addition of the purified thromboplastic protein of beef lung, the evidence of the presence in normal blood of a thromboplastic protein fraction, sedimentable by high speed centrifugation, similar to that isolated from tissue cells is discussed.

BIBLIOGRAPHY

1. Chargaff, E., Moore, D. H., and Bendich, A., J. Biol. Chem., 145, 593 (1942).
2. Chargaff, E., Bendich, A., and Cohen, S. S., J. Biol. Chem., 156, 161 (1944).
3. Chargaff, E., J. Biol. Chem., 161, 389 (1945).
4. Tocantins, L. M., Medicine, 17, 155 (1938).
5. Quick, A. J., The hemorrhagic diseases and the physiology of hemostasis, Springfield (1942).

6. Macfarlane, R. G., Trevan, J. W., and Attwood, M. P., *J. Physiol.*, **99**, 7P (1940–41).

7. Quick, A. J., *Am. J. Med. Sc.*, **201**, 469 (1941).

8. Joules, H., and Macfarlane, R. G., *Lancet*, **1**, 715 (1938).

9. Madison, F. W., and Quick, A. J., *Am. J. Med. Sc.*, **209**, 443 (1945).

10. Pickering, J. W., and Gladstone, R. J., *J. Physiol.*, **59**, p. lxv (1924–25).

11. Lozner, E. L., Jolliffe, L. S., and Taylor, F. H. L., *Am. J. Med. Sc.*, **199**, 318 (1940).

12. Chargaff, E., and Olson, K. B., *J. Biol. Chem.*, **122**, 153 (1937–38).

13. Chargaff, E., *J. Biol. Chem.*, **160**, 351 (1945).

14. Chargaff, E., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **5**, 31 (1945).

15. Ziff, M., and Chargaff, E., *J. Biol. Chem.*, **136**, 689 (1940).

COLORIMETRIC DETERMINATION OF GLYCOGEN

DISADVANTAGES OF THE IODINE METHOD

By DANIEL LUZON MORRIS

(From the Department of Nutritional Research, Mead Johnson and Company, Evansville, Indiana)

Since one of the most widely known properties of glycogen is the red-brown color that it gives with iodine, it is not surprising that attempts have been made to use this color for the comparison of different samples of glycogen (1, 2) or for the measurement of glycogen (3, 4). The experiments reported here indicate, however, that such methods are not very satisfactory for quantitative work: although the color produced with iodine does vary with the concentration of glycogen, it also varies with the temperature, with the concentration of iodine, and even with the glycogen sample.

EXPERIMENTAL

Effect of Glycogen Concentration—Fig. 1 shows the relation between the glycogen concentration and the light absorbed by solutions of constant iodine concentration. The experimental conditions were the following. A solution of corn glycogen (5) was prepared which contained about 2 mg. per ml. Its exact concentration was determined by measurement of the glucose formed after hydrolysis. A measured volume of this solution (0.5 to 5 ml.) was put into a volumetric flask, and water added to make 5 ml. Then, in succession, were added 0.5 ml. of 10 per cent potassium iodide, 5 ml. of 0.01 N potassium iodate, and 5 ml. of 1 N acetic acid solutions. The volume was made up to 25 ml., and the solution was poured into a colorimeter tube standing in a large beaker of water at 22.5° (close to room temperature). After at least 20 minutes, and then at intervals of 10 minutes, the color density was measured with an Evelyn photometer, with a 565 mμ filter. The instrument was set to read 100 per cent transmission with a blank containing iodine but no glycogen. There was no change in the color density over a 20 hour interval if the tubes were kept stoppered to prevent the evaporation of iodine. No correction was made for the light absorption of the glycogen itself, since it was found that such a correction would be less than 1 per cent. The line shown in Fig. 1, which is plotted logarithmically, curves slightly. Similar curves, but differently placed, result if the experimental conditions are varied.

Effect of Temperature—Fig. 2 shows the effect of temperature on the color density of a solution of constant glycogen and iodine concentration. The
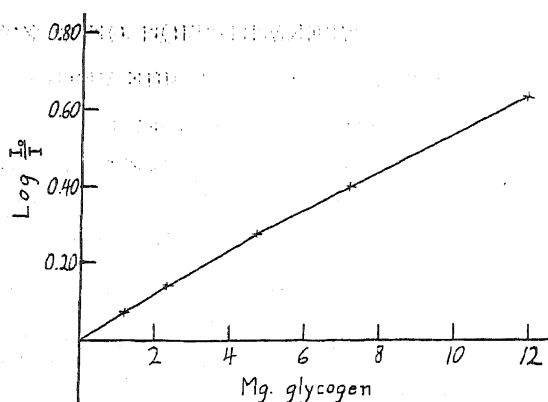
FIG. 1. The color density, at 565 mμ, of solutions containing 0.05 m.eq. of iodine and varying amounts of glycogen in 25 ml. at 22.5°.
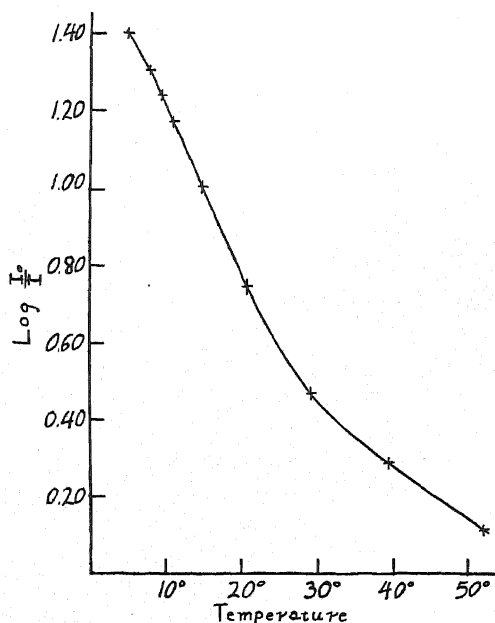

FIG. 2. The color density of a solution containing 11.8 mg. of glycogen and 0.05 m.eq. of iodine in 25 ml. at varying temperatures.

solution contained 11.8 mg. of glycogen and iodine was added in the same way as before. For each reading, colorimeter tubes containing the glyco-gen-iodine solution and an iodine blank were put in a water bath at the

desired temperature and allowed to reach the temperature of the bath. The tubes were then quickly dried, and the color measured within a few seconds. (The color density of the iodine blanks varied by a few per cent over the temperature interval between 5° and 50°; the changes were in the opposite direction from those of the glycogen-iodine complex, *i.e.* the color increased with higher temperature.) The variations with temperature were found to be reversible and perfectly reproducible. It will be seen that over a 5° interval in the normal room temperature range there is a variation of nearly 25 per cent in the value of log I_0/I. Similar variations of color with temperature were observed when the experimental conditions were those of van Wagtendonk, Simonsen, and Hackett (4).

Effect of Iodine Concentration—Fig. 3 shows the effect of iodine concentration on the color produced with glycogen. Here the glycogen concentra-
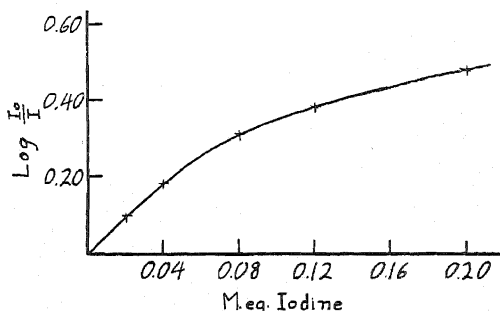
Fig. 3. The color density of solutions containing 3.86 mg. of glycogen and varying amounts of iodine in 25 ml. at 23°.

tion and the temperature were kept constant, and the color density of the glycogen-iodine complex was measured in each case against that of a blank containing iodine at the same concentration.

Effect of Source of Glycogen—One 8.5 mg. sample each of glycogen from corn, from rabbit liver, and commercially obtained[1] was compared under identical conditions. The values for log I_0/I were respectively 0.670, 1.421, and 0.260. Thus the results are greatly affected by variation in the source of the glycogen.

Sumner and Somers Test for Glycogen—A word might be said here about the test for glycogen which was proposed by Sumner and Somers (6). This consists in the addition of a very small amount of iodine to a 0.25 per cent solution of glycogen in half saturated ammonium sulfate solution. By this test they have found a difference between liver and corn glycogen, the first

[1] Pfanstiehl's c.p., stated by the manufacturer to have been prepared from rabbit livers.

giving a yellow and the second a pink color. We have not been able to make this distinction; with either liver or corn glycogen (both purified by treatment with alkali) a salmon-pink color was obtained. On the other hand, Pfanstiehl glycogen gave a pure yellow color. Chargaff and Moore (7), comparing liver glycogen with glycogen from tubercle bacilli, noted that in the Sumner and Somers test the liver glycogen gave a red-brown color, whereas the bacterial glycogen gave a pure yellow color.

DISCUSSION

It has been well known that the color of either a starch-iodine or a glycogen-iodine solution would fade if the solution were heated, and return if cooled. With starch the change in color in the room temperature range is small, and methods such as Nielsen's (8), which use the starch-iodine color for starch determination, are probably subject to only small errors due to changes in temperature. These errors are likely to be much larger with glycogen, and therefore in the use of such methods as those of Jung (3) or of van Wagtendonk *et al.* (4) great care must be taken that all measurements are made at the same temperature. Likewise the variation of the color of the glycogen-iodine complex with iodine concentration is not unexpected in the light of the work of Bates, French, and Rundle (9). In view of this variation, however, the quantity of iodine used in such determinations must be very carefully measured, and precautions taken to avoid evaporation. Finally, it is important that the readings for a given glycogen sample be compared with those of a standard containing glycogen prepared from the same source, by the same method (*cf.* (2)), and preferably by the same investigator. The complexity of these precautions seems to make colorimetric assay of this type of relatively little value when even moderate accuracy is desired.

SUMMARY

The red-brown color formed by glycogen with iodine varies in intensity not only with the concentration of the glycogen but also with the temperature, the iodine concentration, and the source of the glycogen. These variations are so great that extraordinary precautions must be used when the concentration of glycogen is to be determined by the measurement of this color.

It has not been found possible to distinguish between glycogen from animal and vegetable sources by differences in the color produced upon the addition of ammonium sulfate and iodine.

BIBLIOGRAPHY

1. Lazarow, A., *Arch. Biochem.*, 7, 337 (1945).
2. Young, F. G., *Biochem. J.*, 31, 711 (1937).

3. Jung, C., *Compt. rend. Soc. phys. et hist. natur. Genève*, **58**, 137; *Chem. Abstr.*, **38**, 5239 (1944).

4. van Wagtendonk, W. J., Simonsen, D. H., and Hackett, P. L., *J. Biol. Chem.*, **163**, 301 (1946).

5. Morris, D. L., and Morris, C. T., *J. Biol. Chem.*, **130**, 535 (1939).

6. Sumner, J. B., and Somers, G. F., *Arch. Biochem.*, **4**, 7 (1944).

7. Chargaff, E., and Moore, D. H., *J. Biol. Chem.*, **155**, 493 (1944).

8. Nielsen, J. P., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 176 (1943).

9. Bates, F. L., French, D., and Rundle, R. E., *J. Am. Chem. Soc.*, **65**, 142 (1943).

TOCOPHEROL CONTENT OF SKELETAL MUSCLE: COMPARISON OF CHEMICAL AND BIOASSAY METHODS*

BY HANS KAUNITZ AND J. J. BEAVER

(*From the Departments of Pathology, College of Physicians and Surgeons, and of Chemistry, Columbia University, New York*)

The number of determinations of tocopherols in muscle tissue reported in the literature is small. This may be due to the complexity and time-consuming technique of both the chemical and biological methods, as well as to the poor agreement between the two assays. An accurate account of the chemical assay of vitamin E has recently been compiled in Dr. K. C. D. Hickman's laboratory.[1]

The fact that the chemical determinations usually yielded smaller values than the bioassays was probably due to hydrolysis of the extracts to eliminate fats and filtration through an adsorbent earth to separate the tocopherols from carotenes. These procedures were found to be unnecessary if the method of Kaunitz and Beaver (1, 2) for the determination of tocopherols in the presence of fats was applied to muscle extracts.

Extraction of Muscle Tissue

Fig. 1 demonstrates the amounts of fat and tocopherols successively extracted from 97 gm. of human skeletal muscle with 900 ml. of solvent. First, the muscle was emulsified with acetone for 10 minutes in a Waring blendor. The volume was made up with acetone in two centrifuge tubes to 1 liter and permitted to stand with occasional shaking; equilibrium was obtained in 24 hours, a time which could have been considerably shortened with a shaking machine. After centrifuging, the fat, tocopherol, and carotene contents of the supernatant fluid were determined by extracting the acetone water layer three times with 300 ml. of purified Skellysolve B, washing the Skellysolve with distilled water, centrifuging, and evaporating under approximately 20 mm. of Hg pressure under N. The residue was weighed, the carotenes were determined by dissolving an aliquot of the residue in a known volume of Skellysolve, and the $-\log T$ value determined in a Coleman universal spectrophotometer or in a Beckman spectrophotometer. The tocopherols were determined according to the method of Kaunitz and Beaver (1, 2) with modifications to be discussed below. The second extraction of the residue of the muscle tissue was a duplicate of

---

[1] Baxter, J. G., Biological symposia, Lancaster, **12**, in press.

the first; the others were modified by first using one-half acetone and Skellysolve and then one-third acetone and two-thirds Skellysolve; for the eighth and ninth extractions, concentrated HCl was added as indicated in Fig. 1.

In Fig. 2 are shown the results of successively extracting 6700 gm. of beef muscle with 12 liters of solvent for each extraction. The solvent varied from methyl alcohol to a mixture of one-third methyl alcohol and two-thirds Skellysolve. Concentrated HCl was added for the last two extractions as indicated in Fig. 2.
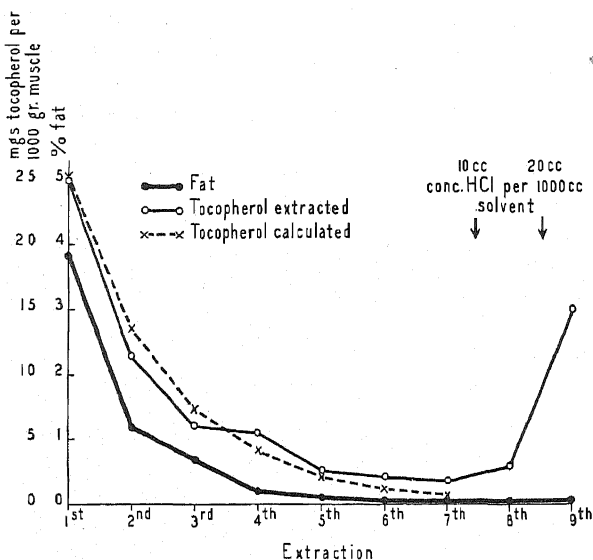
FIG. 1. Total fat and tocopherols obtained by repeated extraction of human muscle.

The data in Figs. 1 and 2 demonstrate that the acetone-Skellysolve mixture removed fat, tocopherol, and carotenes at a more rapid rate than the alcohol-Skellysolve combination, and also that the tocopherol and carotene concentrations roughly parallel the fat concentration curve, except for the first four alcohol concentrations in Fig. 2, in which a relatively higher concentration of tocopherols and carotenes compared to fat was observed. Part of these differences in the rate of extraction is of course due to the change in the solvent-solid ratio.

On the assumption that a distribution coefficient can be calculated between the volume of the emulsified muscle and the volume of the solvent, it is possible to calculate the number of extractions necessary for 98 per cent

extraction of the tocopherols. With a distribution coefficient of 12, the curve indicated in Fig. 1 was obtained. It follows the curve obtained by direct measurements within the experimental error.

On the basis of the above experimental results and calculations, four extractions for at least 24 hours each with a solvent-solid ratio of 20:1, twice with acetone, once with one-half acetone and one-half Skellysolve, and once with one-third acetone and two-thirds Skellysolve, were used as a standard procedure.
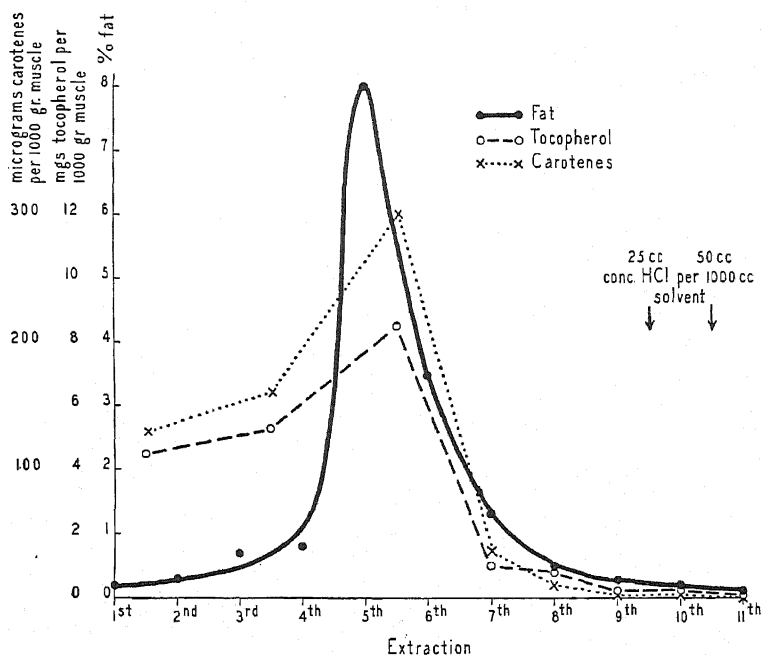
FIG. 2. Total fat, tocopherols, and carotenes obtained by repeated extraction of beef muscle.

Effect of Carotenes and Vitamin A

Since carotenes and vitamin A are always present in the tissue extracts and have reducing properties similar to the tocopherols, various procedures have been described to eliminate these substances.

Previous workers have usually sought to accomplish this by filtration through an adsorbent earth (Emmerie and Engel (3); Devlin and Mattill (4); Mayer and Sobotka (5)), advantage being taken of the fact that carotenes and vitamin A are preferentially adsorbed from benzene solutions. The studies presented here indicate, however, that some tocopherol is

adsorbed on the earth when fat is present.   This difficulty has not been sufficiently appreciated.

In previous papers (1, 2), a determination of tocopherols in the presence of fats was described in which the Merck modification (4) of the Emmerie-Engel iron-bipyridine reagent (3) was used.   Since fats depress the color formed by this reagent with tocopherol, measurements of the absorption
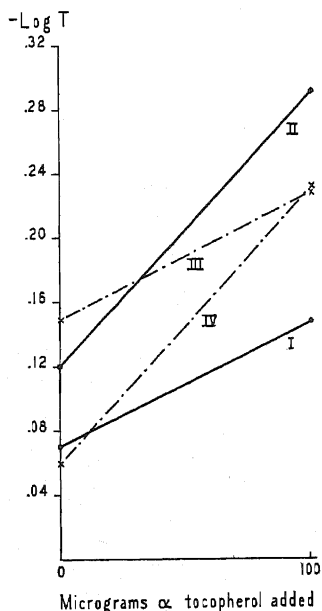
FIG. 3. Influence of filtration through floridin on tocopherols in the presence of fat. Curves I and II, 1 gm. of sesame oil and 1000 γ of synthetic dl-α-tocopherol were dissolved in 20 ml. of benzene.   One-half of the solution was evaporated, and the residue taken up in 5 ml. of Skellysolve.   For Curve II, the benzene mixture was filtered through a 50 × 25 mm. column of HCl-treated floridin, the column washed three times with benzene, the latter evaporated, and the residue treated as for Curve I.   70 γ of α-tocopherol out of 100 γ were recovered (for calculation see the equation in "Procedure").   Curves III and IV, a similar experiment with rat fat.   From Curves III, the presence of 170 γ of α-tocopherol per ml. of Skellysolve can be calculated.   Curve IV indicates recovery of 35 γ after floridin filtration.

coefficient must be made with and without the addition of known amounts of tocopherol.

This procedure is illustrated in Fig. 3 in which Curve I represents the maximum −log T values of the bipyridine-fat-tocopherol mixtures necessary to determine 100 γ of synthetic dl-α-tocopherol[2] in 100 mg. of sesame

[2] We are indebted to Hoffmann-La Roche, Inc., for supplying us with α-tocopherol.

oil. The method is described below. In Curve II are plotted the results obtained for the same tocopherol-sesame oil mixture after filtration with benzene as solvent through floridin,[3] which had been treated according to Devlin and Mattill (4) with concentrated HCl. From the −log T values of Curve I, Fig. 3, the presence of 100 γ of α-tocopherol, as originally added, is calculated from the equation given below (under "Procedure"). Curve II indicated that only 70 γ were present after filtration. Despite the loss of 30 per cent of the tocopherol, the −log T value of the filtered solution, without further tocopherol addition, is greater than that of the original. Since fats, depending on their concentration and physicochemical properties, depress the color (1), the above result indicates that fat was either adsorbed or chemically altered by the floridin.

TABLE I

Influence of Floridin Filtration on Recovery of Tocopherol

Tocopherol used	Amount of fat added	Tocopherol recovered after filtration
γ	*mg.*	*per cent*
110	0	101
60	8 (Rat)	80
60	20 "	78
60	20 "	70
60	40 "	72
214	100 "	52
60	200 "	58
110	480 "	4
104	50 (Sesame oil)	62
428	100 " "	68

Curves III and IV in Fig. 3 present an experiment with rat fat in which 82 per cent of the tocopherol was lost after filtration. The −log T value after filtration is less than that of the original.

Similar experiments, in which two different floridin samples were used and in which the amounts of fat and of tocopherol were varied, are demonstrated in Table I. The fat and tocopherol were dissolved in purified benzene, and filtered through a floridin column that had been filled with nitrogen. After washing the column with additional benzene, the latter was evaporated in an atmosphere of nitrogen, the residue taken up in purified Skellysolve B, and the tocopherol content of this mixture determined according to the method described below. The recoveries ranged from 4 to 80 per cent, with the greatest losses observed when 100 mg. or more of fat had been added to the benzene.

[3] Courtesy of the Floridin Company, Warren, Pennsylvania.

In methods previously used, the muscle extracts were subjected to saponification prior to filtration. The amount of fat present during the filtration was consequently small. One would therefore expect that the losses caused by filtration were not excessive.

These experiments indicate the difficulties encountered when a separation of tocopherols from carotenes is tried in the presence of fats. Since it has been shown previously ((1), p. 654) that tocopherol losses may occur if hydrolysis of the fat is attempted, neither filtration nor hydrolysis seems useful for a quantitative determination of tocopherol in tissue extracts.

When one attempts to determine the reductive capacity of a muscle extract with the iron-bipyridine reagent, accurate results cannot be ob-
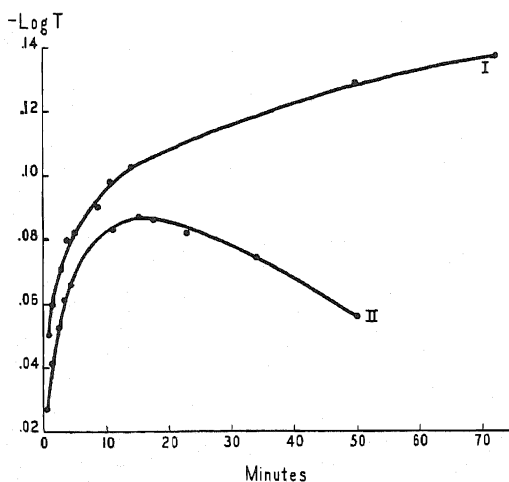
Fig. 4. Curve I, 0.1 ml. of rat muscle extract made to 2 ml. with Skellysolve plus 10 ml. of iron-bipyridine reagent. Curve II, 0.1 ml. of rat muscle extract plus 0.1 ml. of sesame oil made to 2 ml. with Skellysolve plus 10 ml. of iron-bipyridine reagent.

tained because the $-\log T$ value continues to increase for more than 1 hour (Curve I, Fig. 4). If, however, (Curve II) sufficient sesame oil is present in the reacting mixture, a maximum $-\log T$ is reached within 20 minutes. Sesame oil was chosen for these experiments because it usually contains only negligible amounts of reducing substances. Although different samples of the oil produce with the iron-bipyridine reagent different rates of color development for a given amount of tocopherol, or carotenes, the accuracy of the extrapolation method is not affected.

With these facts in mind, correction for the carotenes present in the muscle extract could be made. The determination of the reductive power of carotenes in the absence and in the presence of various fats indicated that

1 γ of carotene is equivalent to 4.8 γ of α-tocopherol; the factors varied from 4.4 to 5.0; since the reductive power of carotenes in a muscle extract does not exceed 10 per cent of the total, the error resulting for the tocopherol from the variations of the carotene factors does not exceed 1 per cent. The total amount of carotenes in the muscle extract was determined by measuring the −log T value of the extract at 4400 A and reading the quantity from a standard curve. Thereafter, 4.8 times the micrograms of carotenes found are deducted from the total reductive power of the extract expressed in micrograms of tocopherol.

A possible error of approximately 2 to 5 per cent is caused by the presence of the normal amounts of vitamin A in the extracts. This estimate is based upon the experimental fact that the reductive power of 1 i. u. of vitamin A was found to be equivalent to 0.24 ± 0.02 γ of α-tocopherol and that 1000 gm. of muscle contain roughly 4000 i. u. of vitamin A. Since it was found that the antimony trichloride method for vitamin A in muscle extracts gave very erratic results, no correction for vitamin A was introduced.

Effect of HCl in Extraction

In experiments reported by Hines and Mattill (6), it was found that, after extraction of rat muscle with alcohol-Skellysolve, further extraction with alcohol-Skellysolve containing HCl brought about an additional yield of 20 to 30 per cent of reducing substance, which they were inclined to believe may have been tocopherol.

As can be seen in Fig. 1, the addition of HCl to the solvent resulted in a further extraction of reducing substance from human muscle, while Fig. 2 shows no further yield from beef muscle under similar conditions.

In these experiments, it was noted that additional reducing substances in the extracts occurred after acid hydrolysis only when a purplish discoloration developed, which was observed especially with rat muscle. Only the colored extracts gave increases up to 25 per cent of the total reducing substances, while the colorless extracts gave none. It therefore seems probable that these additional reducing substances are not tocopherol, and this assumption is supported by the fact that there is good agreement between Mason's (7) bioassays on whole muscles and the values obtained by chemical analysis of muscle extracts without acid hydrolysis (see below).

Method

Reagents—

1. Skellysolve B. Purified by twice shaking with concentrated sulfuric acid, washing with water, 10 per cent Na_2CO_3, 4 times with water, drying over anhydrous Na_2SO_4, and distilling in an all-glass apparatus. A 2 ml. residue from 2 liters gave a −log T value of less than 0.002 with the iron-

bipyridine reagent when compared with 2 ml. taken from the solvent as used.

2. Acetone. Baker's or Merck's reagent grade.

3. Sesame oil. A sample kept in the laboratory for at least several weeks was used. 0.1 ml. of the oil made to 2 ml. with Skellysolve should have a $-\log T$ value of less than 0.002 with 10 ml. of the iron-bipyridine reagent. If the $-\log T$ value is higher, correction has to be made.

4. Iron-bipyridine reagent, as recommended by Merck and Company to Devlin and Mattill (4). 250 mg. of $FeCl_3 \cdot 6H_2O$ (reagent grade) and 500 mg. of α, α'-bipyridine are made to 1 liter with glacial acetic acid.

5. Synthetic dl-α-tocopherol.

Procedure—30 to 50 gm. of muscle, weighed to 0.2 gm., are emulsified with about 100 ml. of acetone in a Waring blendor for 6 minutes and washed into a centrifuge tube with an additional 350 ml. of acetone. Determinations can be made with 5 gm. of muscle but with a larger error. After 24 hours, the tube is centrifuged, the residue once more extracted with acetone, once with one-half acetone and one-half Skellysolve, and once with one-third acetone and two-thirds Skellysolve. The combined extracts are transferred to a 3 liter separatory funnel, and water is added until two distinct layers are formed. The Skellysolve phase is saved, the watery phase is twice extracted with 200 ml. of Skellysolve, and the combined Skellysolve extracts are washed three times with 500 ml. of distilled water, centrifuged, and evaporated in an all-glass apparatus under reduced pressure in an atmosphere of nitrogen. The residue is washed into a 25 ml. flask with approximately 20 ml. of Skellysolve; 1 to 2 ml. of sesame oil (according to its color-depressing properties) is added and the volume made to 25 ml. with Skellysolve. The mixture should be slightly yellow and entirely clear. In rare instances, a brown, strongly reducing pigment is noted which interferes with the determination. The pigment can often be removed by washing the Skellysolve extract with a 2 per cent Na_2CO_3 solution.

For the carotene determination, the $-\log T$ value of the optically clear solution is determined in a Coleman universal spectrophotometer or in a Beckman spectrophotometer with Skellysolve as blank at 4400 A. The concentration is read from a calibration curve.[4]

Two standard solutions containing 40 to 50 and 80 to 100 γ of synthetic dl-α-tocopherol per ml. of Skellysolve (determined to 1 γ) are prepared.

Aliquots of the muscle extract of 1 ml. each are pipetted into three test-tubes; to one of these is added 1 ml. of Skellysolve, and to each of the other two 1 ml. of the standard tocopherol solutions. To each test-tube are

---

[4] We are indebted to Dr. Dorothy Andersen and Mrs. Helen Kennedy for a carotene calibration curve.

added 10 ml. of iron-bypyridine reagent; 2 ml. of Skellysolve containing the same amount of the same sesame oil as the unknown plus 10 ml. of iron-bipyridine reagent serve as a blank. The test-tubes are kept in the dark. Approximately every minute a $-\log T$ reading is taken, starting with the sample to which the higher tocopherol concentration was added. As soon as the maximum reading is reached, the second tocopherol-containing sample is examined, and finally the sample to which no tocopherol had been added. This procedure is possible because it was found experimentally that the maximum reading appears fastest in the sample with the highest tocopherol concentration. The dilutions of the extracts should be such that none of the $-\log T$ readings exceeds 0.4.

TABLE II

Tocopherol Content of Skeletal Muscle (Chemical Analysis)

Species	Tocopherol per 1000 gm. wet muscle	Species	Tocopherol per 1000 gm. wet muscle
	mg.		*mg.*
Human	18	Rat	17
	20		18
	21		18
	23		19
	24		21
	25		24
	29		29
	29		
	30	Cattle	20
	33		
	48*		

* From a case of myasthenia gravis.

The uncorrected value, x, for the number of micrograms of tocopherol per ml. of diluted extract is given by the expression $x = (e_1 n)/(e_2 - e_1)$ where e_1 represents the $-\log T$ value of the unknown, e_2 that of the unknown plus n γ of tocopherol, and n the number of micrograms of tocopherol added. For each microgram of carotene per ml. of diluted extract, 4.8 is subtracted from the value for x. All results are finally computed on the basis of mg. of tocopherol per 1000 gm. of muscle.

With different tocopherol additions and different extract concentrations, the maximum errors observed did not exceed ± 10 per cent.

Results

Muscles from humans, rats, and in one case from cattle, have been examined (Table II). For the experiments on humans, the psoas muscle

was used as soon as possible after the death of the patient. No attention has so far been paid to the cause of death. The rats used had been kept on Rockland pellets throughout life. Any kind of skeletal muscle was used.

The results indicate values considerably higher than those reported by Karrer, Jaeger, and Keller (8), who found 5.9 mg. per 1000 gm. of cattle muscle, and by Hines and Mattill (6), who reported 7.5 mg. of tocopherol per 1000 gm. of rat muscle. In both instances, filtration and saponification of the extracts had been performed and no bioassays were carried out by the authors.

Bioassays—In order to check the above values, bioassays were carried out. Albino rats, whose ancestry had been kept for seven generations on an Evans-Burr diet, were mated, and they and their offspring were placed on the day of birth of a litter on a diet[5] consisting of lard 10 parts, casein (crude) 30 parts, cerelose 54 parts, celluration 2 parts, salt mixture[6] 4 parts, thiamine chloride 2 mg. per kilo, riboflavin 4 mg. per kilo, pyridoxine 4 mg. per kilo, nicotinic acid 100 mg. per kilo, choline 1000 mg. per kilo, vitamin K 4 mg. per kilo, p-aminobenzoic acid 300 mg. per kilo, calcium pantothenate 10 mg. per kilo, and percomorph oil[7] 0.2 ml. per kilo.

The females were used for bioassays after they had reached a weight of 170 gm. or more. They were left for 5 days with the male. Pregnancy occurred in 97 times out of 127 mating experiments in rats of below $6\frac{1}{2}$ months of age. In forty-three tests carried out when these rats were $6\frac{1}{2}$ to $8\frac{1}{2}$ months old, pregnancy was observed thirty-three times. Thereafter, the precentage of pregnancies declined considerably. Only rats of below $6\frac{1}{2}$ months of age were used for the experiments reported in this paper. In the majority of the tests, the desired amount of tocopherol and a comparable dose of muscle extract were fed to a pair of litter mates.

Table III demonstrates the number of litters and of resorptions observed with different tocopherol supplements at different age levels. When the percentage of litters was plotted against the tocopherol supplement, the resulting curves suggested a "mean fertility dose" of about 1.1 mg. of synthetic α-tocopherol for the younger group and about 1.3 mg. for the older ones. In view of the fact that the members of the older group had previously received doses of from 1 to 2 mg. of tocopherol, it was concluded that those previous supplements had no measurable effect on the subsequent tests. The results also indicated that the tocopherol requirements

[5] The diet used in these experiments was developed in cooperation with Dr. Charles Slanetz, Department of Animal Care, College of Physicians and Surgeons, Columbia University. We gratefully acknowledge his cooperation in the bioassay work.

[6] Per cent composition: NaCl 4.35, $MgSO_4$ 13.70, NaH_2PO_4 8.72, K_2HPO_4 23.98, CaH_2PO_4 13.58, ferric citrate 2.97, Ca lactate 32.70.

[7] We are indebted to the Abbott Laboratories for the synthetic vitamins.

of the older group were not considerably higher than those of the younger group.   It was therefore decided to combine the results of the two groups

TABLE III

Mating Experiments with Tocopherol-Deficient Female Rats at Different Ages

α-Tocopherol supplement	Below 4 mos.		4–6½ mos.	
	No. of resorptions	No. of litters	No. of resorptions	No. of litters
mg.				
0.0	11	0	8	0
0.5	6	0	2	0
0.75	2	2	5	3
1.0	4	4	3	2
1.5	3	5	2	2
2.0			·2	5
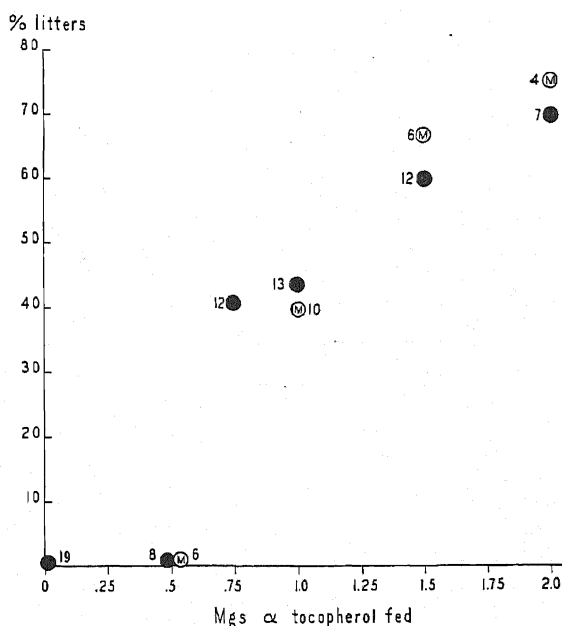
FIG. 5. Bioassays with graded doses of tocopherol.   ● represents the results with synthetic *dl*-α-tocopherol, ○ the results with muscle.   The figures close to the symbols give the numbers of experiments at the respective levels.

for comparison with the animals that had received muscle extract.   The latter were proportionally of the same age as the controls.

On the 5th day after mating had begun, the male was removed and 30

gm. of the experimental diet containing the tocopherol supplement were offered, which the rats finished in 2 to 3 days. Particular care was taken that the cages were clean when the tocopherol-containing food was offered, and that the rats had completely finished it before the subsequent feeding. The weight of the animals was taken daily, beginning 15 days after mating, and they were examined daily for the presence of a vaginal plug. Only when a vaginal plug was followed by a weight gain and then by weight loss, was it taken as proved that resorption had occurred. The birth of a living litter surviving for at least 1 day was accepted as a positive result.

For the bioassays of the muscle extracts, the tocopherol content of the extracts was calculated according to the chemical test. The extract was diluted with experimental diet, so that 30 gm. contained the desired amount of tocopherol. Otherwise, the animals were treated exactly as the controls that received synthetic *dl-α*-tocopherol.

No "first litter fertility" was observed (Fig. 5), and at least 2 months were allowed to elapse between tests on the same female; if a litter was born, the rat was not mated for 2 months after the birth of the young.

Fig. 5 demonstrates the results of the bioassays. No litters were observed in eight pregnant rats with 0.5 mg. of synthetic *dl-α*-tocopherol, or in six that had received muscle extract supposedly containing 0.5 mg. of tocopherol. With 0.75, 1.0, 1.5, and 2.0 mg., the number of litters rose from 38 to 70 per cent. The results suggest a "mean fertility dose" of 1.25 mg. of synthetic *dl-α*-tocopherol for this particular colony of rats.

With muscle extract, the tocopherol content of which had been determined by the chemical method, the results were found to be within ±10 per cent of those obtained with synthetic *dl-α*-tocopherol.

DISCUSSION

Mason (7) used whole rat muscle as substrate for tocopherol bioassays. With 60 gm. of muscle of rats kept on dog chow, 50 per cent positive results were observed.

In these experiments, the chemical analysis revealed values of 17 to 30 mg. of tocopherol per 1000 gm. of muscle; the bioassay indicated a "mean fertility dose" of 1.25 mg. of tocopherol, which suggests that 40 to 70 gm. of muscle would yield 50 per cent positive responses. This is in good agreement with Mason's findings, and it is an indication of the fact that complete extraction of the tocopherols is achieved without hydrolysis of the tissue.

The circumstance that the bioassays, for which synthetic *dl-α*-tocopherol had been used as standard, agree with the chemical analyses indicates that the bulk of the reducing substances in a Skellysolve extract of muscle, except for carotenes and vitamin A, is tocopherols and that the bulk of the

tocopherols consists of α-tocopherol. If any considerable amount of the β or γ form were present, no agreement between chemical and bioassay could be expected in view of the much lower biological activity of the latter.[8]

The agreement between chemical and bioassay methods also suggests that the bulk of the α-tocopherol in the muscle is present in its free, not esterified form, because tocopherol esters would not react with the iron-bipyridine reagent.

SUMMARY

1. A chemical method for the determination of tocopherols in skeletal muscle is described.

2. In human and rat muscle, 17 to 30 mg. of tocopherol per 1000 gm. of wet muscle were recovered.

3. The chemical findings were checked by bioassays; agreement within ±10 per cent between the two methods could be demonstrated.

4. The conclusion seems permissible that the bulk of the reducing substances in a Skellysolve extract of muscle consists, except for carotenes and vitamin A, of non-esterified α-tocopherol.

BIBLIOGRAPHY

1. Kaunitz, H., and Beaver J. J., *J. Biol. Chem.*, **156**, 653 (1944).
2. Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, **156**, 661 (1944).
3. Emmerie, A., and Engel, C., *Rec. trav. chim. Pays-Bas*, **59**, 246 (1940).
4. Devlin, H. B., and Mattill, H. A., *J. Biol. Chem.*, **146**, 123 (1942).
5. Mayer, G. G., and Sobotka, H., *J. Biol. Chem.*, **143**, 695 (1942).
6. Hines, L. R., and Mattill, H. A., *J. Biol. Chem.*, **149**, 549 (1943).
7. Mason, K. E., *J. Nutr.*, **32**, 71 (1942).
8. Karrer, P., Jaeger, W., and Keller, H., *Helv. chim. acta*, **23**, 464 (1940).
9. Harris, P. L., Jensen, J. L., Joffe, M., and Mason, K. E., *J. Biol. Chem.*, **156**, 491 (1944).

---

[8] Dr. P. L. Harris suggested a different explanation (9). Harris, Jensen, Joffe, and Mason have found that the biological activity of natural α-tocopherols is 50 per cent greater than that of the synthetic α-tocopherol. It is therefore possible that mixtures of natural α-tocopherol with the β and γ forms could give the same effect as synthetic α-tocopherol. While this possibility cannot be ruled out at present, it does not seem probable that mixtures containing the β and γ forms in any considerable amount would give agreement of the chemical and bioassay methods used for these experiments.

SYNTHESIS OF ACETIC ACID CONTAINING ISOTOPIC CARBON IN THE METHYL GROUP*

By H. S. ANKER

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York; and from the Department of Biochemistry, University of Chicago, Chicago)

Studies of intermediary metabolism by means of the isotopes of carbon require methods for the synthesis of compounds that contain the isotope in a known position of the carbon chain. It is possible to introduce isotopic carbon into a carboxyl group by the interaction of a Grignard compound with isotopic carbon dioxide. It is also possible to synthesize compounds via acetylene, with the isotope in two adjacent carbon atoms (1). However, the introduction of the isotope into other positions requires methods not ordinarily used in organic syntheses. The problem is further complicated by the fact that isotopic carbon is available only in the form of carbon dioxide or of sodium cyanide.

The present report describes a synthesis for acetic acid, with the isotope in the methyl group only (C^*H_3COOH; 2-C*-acetic acid), starting from sodium cyanide. Isotopic sodium cyanide is converted into zinc cyanide and condensed with phenol to yield p-hydroxybenzaldimine hydrochloride. The crude product is dissolved in concentrated hydrochloric acid and reduced by amalgamated zinc and hydrochloric acid. The resulting cresol is then oxidized by chromic acid to yield acetic acid. By this procedure the carbon atom of the methyl group of the acetic acid is derived from that of the original cyanide, while the carbon atom of the carboxyl group is derived from one of the carbon atoms of the phenol. Isotope analyses of the acetic acid and of the products of decarboxylation of lithium acetate, *i.e.* acetone and carbon dioxide, confirm this, the acetone containing the calculated isotope content, about four-thirds of that of the acetic acid, while the carbon dioxide contains no excess of isotope.

EXPERIMENTAL

p-Hydroxybenzaldimine Hydrochloride (2)—5.9 gm. of zinc cyanide (3), 0.1 gm. of potassium chloride (4), 13 gm. of phenol, and 130 cc. of dry ether are mixed in a 500 cc. 3-neck flask, equipped with inlet tube, stirrer, and reflux condenser with a drying tube. Under stirring, dried hydrogen chloride gas is introduced at a rapid rate. After a few minutes the zinc cyanide dissolves and after about 20 minutes a liquid separates which gradually solidifies. After about 1 hour the mixture is saturated with hy-

drogen chloride, which begins to appear at the outlet of the drying tube. For 3 more hours hydrogen chloride gas is introduced at a reduced rate. During this procedure, the reaction vessel should be protected from direct daylight. The precipitated crystals which consist mainly of *p*-hydroxybenzaldimine hydrochloride and a small amount of *o*-hydroxybenzaldimine hydrochloride (2) are separated from the solution by decanting and are washed three times with dry ether.

p-Cresol (*5*)—60 gm. of amalgamated zinc (mossy or turnings) and 120 cc. of hydrochloric acid (1:1) are heated to boiling. The hydroxybenzaldimine hydrochlorides are dissolved in 60 cc. of concentrated hydrochloric acid and this solution added dropwise over 1 hour to the rapidly refluxing reducing mixture. After all of the solution has been added, the mixture is refluxed for 3 more hours. It is filtered through glass wool, diluted with 3 to 5 volumes of water, and extracted several times with ether. The ether is evaporated to yield *p*-cresol, admixed with small amounts of *o*-cresol. Yield, 8.2 gm. (75 per cent).

Acetic Acid (*6*)—2 gm. of *p*-cresol are dissolved in 25 cc. of concentrated sulfuric acid and added to a mixture of 30 gm. of chromium trioxide dissolved in 25 cc. of water and 150 gm. of ice. After standing for 12 hours at room temperature, 75 cc. of concentrated sulfuric acid are added and the mixture refluxed for 3 hours. The acetic acid is separated from the reaction mixture by steam distillation. This requires about 1 hour. The distillate is treated with an excess of silver carbonate, filtered hot, and the silver acetate is crystallized from the concentrated filtrate. The yield is 50 to 70 per cent, as determined by titration of an aliquot with standard sodium hydroxide solution, before the addition of silver carbonate.

Decarboxylation (*7*)—0.5 gm. of recrystallized silver acetate is dissolved in sufficient hot water and an equal molar amount of lithium bromide is added. The precipitated silver bromide is filtered off, the filtrate evaporated to dryness, and the residue dried over phosphorus pentoxide *in vacuo*. About 80 mg. of the lithium salt were heated in a stream of nitrogen (8) to about 400° and the acetone distilling over condensed in a tube cooled by dry ice. The trap was then inserted into a micro combustion train and the carbon dioxide formed by combustion precipitated as barium carbonate, from a concentrated barium hydroxide solution. The residual lithium carbonate in the decarboxylation vessel was dissolved in dilute hydrochloric acid and the carbon dioxide liberated swept by a nitrogen stream into concentrated barium hydroxide solution and also precipitated as barium carbonate.

Isotope Distribution—In an experiment in which the sodium cyanide used as starting material contained an excess of 0.2 atom per cent C^{13} the acetic acid prepared had an excess of 0.093 per cent C^{13}, *i.e.* 0.186 per cent excess

C^{13} in the methyl carbon. The carbon dioxide obtained by decarboxylation did not contain any excess C^{13} (+0.01 per cent; −0.012 per cent), while the acetone obtained contained an excess of 0.121 per cent C^{13}, *i.e.* 0.183 per cent excess C^{13} in the methyl carbon in good agreement with the value obtained for acetic acid.

SUMMARY

Acetic acid has been synthesized containing isotopic carbon in the methyl group only.

The average yield of acetic acid based on the amount of sodium cyanide used as starting material is greater than 50 per cent.

BIBLIOGRAPHY

1. Cramer, R. D., and Kistiakowsky, G. B., *J. Biol. Chem.*, **137**, 549 (1941). Lorber, V., Lifson, N., and Wood, H. G., *J. Biol. Chem.*, **161**, 411 (1945).
2. Gattermann, L., *Ann. Chem.*, **357**, 318 (1907). Houben, J., Die Methoden der organischen Chemie, Leipsic, **3**, 543 (1930).
3. Adams, R., and Levine, I., *J. Am. Chem. Soc.*, **45**, 2373 (1923). Adams, R., and Montgomery, E., *J. Am. Chem. Soc.*, **46**, 1518 (1924).
4. Arnold, R. T., and Sprung, J., *J. Am. Chem. Soc.*, **60**, 1699 (1938).
5. Clemmensen, E., *Ber. chem. Ges.*, **47**, 61 (1914). Adams, R., Organic reactions, New York, **1**, 164 (1942). Read, R. R., and Wood, J., Jr., in Organic syntheses, New York, **20**, 57 (1940).
6. Kuhn, R., and L'Orza, F., *Z. angew. Chem.*, **44**, 847 (1931).
7. Wood, H. G., Werkman, C. H., Hemingway, A., Nier, A. O., and Stuckwisch, C. G., *J. Am. Chem. Soc.*, **63**, 2140 (1941).
8. Krönig, W., *Z. angew. Chem.*, **37**, 667 (1924).

NITROGEN METABOLISM IN PYRIDOXINE INSUFFICIENCY

By WINTHROP W. HAWKINS, M. L. MacFARLAND, AND E. W. McHENRY

(From the Department of Public Health Nutrition, School of Hygiene, University of Toronto, Toronto, Canada)

A relation of vitamin B_6 compounds to protein metabolism appears to be established, although the precise mode of action is not yet known. In 1941 McHenry and Gavin (1) reported that pyridoxine was necessary for the synthesis of fat from protein in rats and suggested that this vitamin was essential for the metabolism of protein. Cerecedo and Foy (2) found that pyridoxine deficiency in rats could be accelerated in onset and increased in severity by maintenance of the animals on a high protein diet. Proof of the relation of pyridoxine to protein metabolism has been obtained by the observations of Schlenk and Snell (3) and of Lichstein *et al.* (4) relating the vitamin to enzyme systems. Using another approach, Sure and Ford (5) reported inconclusive results from a study of nitrogen metabolism. This paper describes an investigation of the effects of pyridoxine depletion upon nitrogenous metabolites in rats and dogs.

Studies on Rats

Method—Albino rats of the Wistar strain, reared in the colony of the Connaught Medical Research Laboratories, were used. In part of the study rats with an initial weight of about 100 gm. were employed; in other cases the initial weight was approximately 135 gm. All rats were housed in individual screen bottom cages with water supplied *ad libitum*. A high protein diet containing vitamins A and D similar to that described by McHenry and Gavin (1) was used throughout. B vitamins were given by subcutaneous injection in the following amounts per rat per day: thiamine chloride 25 γ, riboflavin 25 γ, niacin 100 γ, calcium pantothenate 100 γ, choline chloride 10 mg. Control animals also received 40 γ of pyridoxine hydrochloride per day.

Blood was obtained from the exposed heart after the administration of nembutal. Blood constituents were determined by the following procedures: urea, Barrett (6); amino nitrogen, Folin as modified by Danielson (7); uric acid, Benedict as described by Todd and Sanford (8); allantoin, Young *et al.* (9); creatine plus creatinine, Peters (10); total keto acid, Lu (11); non-protein nitrogen, by nesslerization of a trichloroacetic acid or tungstic acid filtrate after heating with sulfuric acid and hydrogen peroxide. All procedures, except that for amino nitrogen, were adapted so that a Coleman spectrophotometer could be used for color estimations.

Fasting Blood Levels of Protein Metabolites in Pyridoxine-Depleted and Control Rats—Rats were maintained on the experimental regimen for 3 to 5 weeks before removal of blood for analysis. In all cases depleted rats lost about 20 per cent in weight, while controls gained about the same amount. Determinations of uric acid and allantoin were made on one sample of blood, as was the case with urea and keto acids. Because of the quantity of blood required and the small amount available, it was necessary to use separate groups of rats for all other analyses. Every effort was made to have all groups comparable with regard to sex, body weight, and treatment. All animals were fasted 16 hours before withdrawal of blood. The results are shown in Table I.

TABLE I

Average Content of Protein Metabolites in Rat Blood

Metabolite	Control		Pyridoxine-deprived	
	No. of rats	Average and s.d.	No. of rats	Average and s.d.
		mg. per cent		*mg. per cent*
Amino nitrogen	26	7.1 ± 0.3	32	7.4 ± 0.3
Uric acid	15	1.8 ± 0.06	13	1.7 ± 0.04
Allantoin	15	7.7 ± 0.3	13	7.6 ± 0.3
Non-protein nitrogen	40	52.5 ± 1.06	34	61.5 ± 2.0
Urea	22	50.0 ± 1.63	19	82.0 ± 4.1
Creatine + creatinine	12	2.6 ± 0.13	13	3.6 ± 0.2
Total keto acids	13	1.1 ± 0.15	16	1.0 ± 0.13

The only significant difference found between depleted and control rats was a higher level of urea (and consequently of non-protein nitrogen) in the depleted animals. No increases were observed in hemoglobin or in erythrocytes, indicating that hemoconcentration was not a factor.

Changes in Blood Urea Levels after Test Dose of Amino Acids—Because of the apparent relation of pyridoxine to the level of blood urea, investigations were made of the effect of a test dose of amino acids upon the blood urea of control and depleted rats. Each rat received intraperitoneally 60 mg. of nitrogen contained in 5 cc. of a 10 per cent solution of casein hydrolysate (amigen, kindly supplied gratis by Mead Johnson and Company). Groups of twenty-five to thirty rats were maintained on the same regimen as in Experiment 1, but the average initial weight was 135 gm. After animals had been maintained thus for 3 weeks, they were fasted 16 hours, casein hydrolysate was administered, and at intervals groups of three to six animals were killed to obtain blood. Because sig-

nificant changes were observed in Experiment 1 only with regard to urea and non-protein nitrogen, analyses in Experiment 2 were limited to those constituents. · Results are shown in Fig. 1. A duplicate experiment gave entirely comparable data.

It will be observed from Fig. 1 that depleted rats showed a greater and more sustained increase in blood urea. No such change was observed in other depleted animals after an injection of the same volume of isotonic
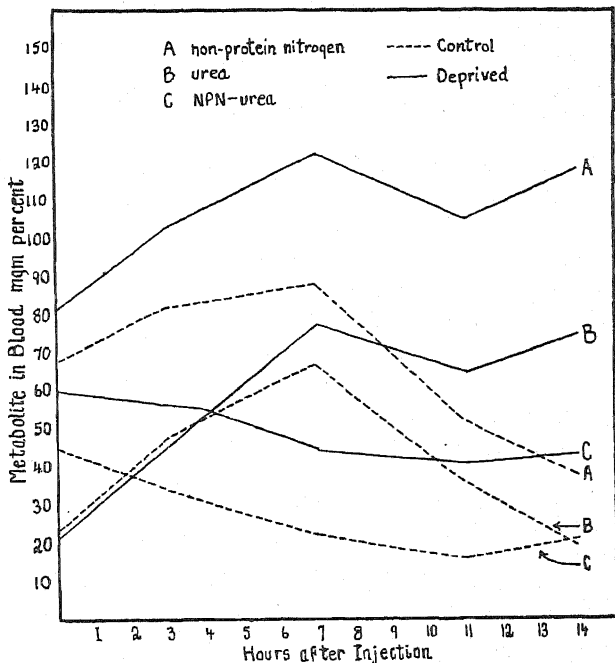

FIG. 1. Changes in urea and non-protein nitrogen in the blood of rats after a test dose of amino acids.

saline, nor in normal animals maintained on a diet of fox chow (Toronto Elevators) after the administration of casein hydrolysate.

In another experiment the load of amino acid nitrogen was supplied by injecting 5 cc. of 7.6 per cent *dl*-alanine in distilled water. Changes in blood urea in both control and depleted animals were entirely comparable to those found after the administration of casein hydrolysate. Consequently, these data are not included.

Studies on Dogs

More information was sought regarding the rôle of pyridoxine in protein metabolism by studying the blood levels and the excretion of various me-

tabolites in dogs with and without the vitamin. The microcytic hypochromic anemia, characteristic of pyridoxine insufficiency in dogs, was used as a criterion of depletion. Three dogs, two adult and one pup, were maintained on a diet with the following percentage composition: casein 42, sucrose 35, lard 9, corn oil 3, bone meal 2, salt mixture 2,[1] cod liver oil 1, liver fraction 3, agar 3. The liver fraction was an alcohol-soluble material prepared in the Connaught Medical Research Laboratories. It was treated with Super Filtrol (Filtrol Corporation) to reduce the pyridoxine content from 40 to 1 γ per cc. The total amount of pyridoxine in the diet was negligible. The following vitamin supplements were added daily to the diet of each dog: thiamine chloride 1.5 mg., riboflavin 1.5 mg., calcium pantothenate 6 mg., niacin 30 mg., choline chloride 750 mg., inositol 1 gm. One adult dog also received 1.5 mg. of pyridoxine hydrochloride.

Samples of urine and feces were collected for 3 day periods at intervals of about 2 weeks. Analyses were made on pooled material for each period with the following procedures: total nitrogen, macro-Kjeldahl; urea and ammonia in urine, Van Slyke and Cullen (12); amino nitrogen in urine, Folin as modified by Danielson (7); uric acid in urine, Benedict and Franke (13); allantoin in urine, Young and Conway (14); creatinine in urine, Folin (15).

Samples of venous blood were withdrawn at different periods for hematological and chemical studies. Procedures used for metabolites were those listed for investigations with the rat. Hemoglobin was measured by the cyanmethemoglobin method of Collier (16). Hematocrit, erythrocyte counts, leucocyte counts and differentials, and reticulocyte estimations were accomplished by standard methods.

The young dog deprived of pyridoxine developed only a mild anemia and difficulties were found in obtaining authentic samples of urine. Consequently, data from this dog are not included. The other dog not receiving pyridoxine developed a marked anemia which responded promptly to administration of the vitamin.

To abbreviate the presentation of data, Table II has been restricted to significant findings. Data have been selected as characteristic of certain phases. These show a marked decrease in hemoglobin and hematocrit with a prompt recovery after pyridoxine hydrochloride was supplied in the 25th week at a level of 1.5 mg. per day. At no time were there any significant changes in blood composition. Coincident with the appearance of insufficiency, as indicated by anemia, there was a significant increase in the excretion of urea, ammonia, and uric acid. To economize in space, only three sets of values for the control dog are shown. While there was some variability in the quantities of metabolites, at no time did the control

---

[1] Salts 40 (Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **56**, 362 (1923)).

TABLE II

Observations on Pyridoxine Deprivation in Dogs

Dog	Wk.	Blood levels									Urine output per day					
		Hemoglobin	Hematocrit	Keto acid	Urea	Uric acid	Allantoin	Creatine + creatinine	Amino N	Non-protein N	Urea	Ammonia N	Uric acid	Allantoin	Creatinine	Amino N
		gm. per cent		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*gm.*	*gm.*	*mg.*	*gm.*	*mg.*	*mg.*
Pyridoxine-deprived	1	17.2	52.5	0.6	34.6	1.7	3.8		6.1		9.6	0.13	27.7	1.7	302.0	528.2
	5	16.4	51.4	0.9	42.0	1.8	5.5	1.5	6.7	30.1	11.0	0.09	35.6	2.0	359.0	259.9
	12	14.3	47.6	1.6	27.2	1.5	5.9		6.8	37.4	25.8	1.04	66.5	2.8	357.0	296.3
	17	13.8	44.7	0.6	32.0	1.4	4.2	2.3	6.9	43.5	28.1	1.07	64.8	2.6	444.0	294.8
	24	10.7	28.4		36.7	0.8	3.5		8.6	36.4	22.1	1.09	71.5	2.4	455.0	325.2
	25	8.5	42.2		32.0	1.0	4.8				23.6	1.18	80.4	2.5	504.0	
	26	12.0	48.3			0.8	5.8	1.6	7.2	44.6	19.6	1.02	32.9	2.0	434.0	236.9
	28	13.8	51.0	1.3	32.0	0.9	3.0		7.1	44.4	17.3	0.74	44.2	1.8	384.0	421.2
	29	14.5			48.3											
Control	Initial	15.5	48.8	1.7	47.0	2.1	5.8	1.9	4.9	37.4	8.8	0.2	15.4	1.3	214.0	257.0
	Highest* or lowest	14.7	45.6	1.7	66.0	2.1	6.6	2.4	7.1	58.2	15.2	0.7	34.3	1.7	344.0	350.0
	Final	16.4	53.0	1.0	57.0	1.3	2.4	1.9	6.3	50.0	9.5	0.4	18.2	0.9	252.0	212.0

* Lowest in the case of hemoglobin and hematocrit, highest in the others.

animal show an anemia or a marked increase in the excretion of urea, ammonia, and uric acid.

DISCUSSION

In the comparison between rats given pyridoxine and those deprived of this vitamin, maintained on a high protein diet, the significant changes in blood levels of metabolites were the increases in urea and in non-protein nitrogen in the deprived animals. Such increases might be due to increased catabolism of amino acids, to diminished excretion of urea, to blood concentration, or to a failure in anabolic processes involving transamination. The explanation of blood concentration is unlikely since there was no increase in hemoglobin or in several other constituents. The final explanation, a possible failure in transamination, is consistent with the observations of Snell et al. (3) and of Gunsalus et al. (4) relating pyridoxine to transaminase.

In the case of rats deprived of pyridoxine and given loads of casein hydrolysate or of alanine, the sustained rise in urea would be expected from the first observations and was in harmony with the transaminase explanation of pyridoxine action.

Data regarding urea excretion are not available from the rat studies. The pyridoxine-deprived dog showed a significant increase in urea excretion and, also, in the urinary content of ammonia, uric acid, and creatinine. Results of vitamin insufficiencies in two species are not always comparable and, in this case, there are differences. The deprived rat showed an increase in blood urea, a change which was not seen in the dog, which did have an increased excretion of urea. It could be said that pyridoxine deprivation did not inhibit urea formation in either species. It is possible that urea was not normally excreted in the deprived rat but was in the dog, thus preventing an increase in blood urea. The data lead to the opinion that pyridoxine insufficiency causes an interference with nitrogen utilization but does not interfere with the formation of urea. This, again, may be concordant with the transaminase explanation of pyridoxine action.

Observations on humans have not yet elicited definite signs of pyridoxine deficiency. If pyridoxine functions in an essential enzyme system, signs of such deficiency might be anticipated. The present observations suggest that studies of nitrogen metabolites might provide signs of pyridoxine insufficiency and that the use of a test load of amino acids might be advantageous in such studies.

SUMMARY

Deprivation of pyridoxine in rats fed a high protein diet caused an increase in the fasting blood levels of urea and of non-protein nitrogen. Ad-

ministration of casein hydrolysate or of alanine to deprived rats caused a marked and sustained increase in blood urea. A dog deprived of pyridoxine developed a microcytic, hypochromic anemia and showed an increased urinary output of urea, ammonia, uric acid, and creatinine. These observations are consistent with the transaminase explanation of pyridoxine action. They suggest signs of pyridoxine insufficiency which might be sought in man.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **138**, 471 (1941).
2. Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, **5**, 207 (1944).
3. Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, **157**, 425 (1945).
4. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945).
5. Sure, B., and Ford, Z. W., *J. Nutr.*, **24**, 405 (1942).
6. Barrett, J. F., *Biochem. J.*, **29**, 2442 (1935).
7. Danielson, I. S., *J. Biol. Chem.*, **101**, 505 (1933).
8. Todd, J. C., and Sanford, A. H. Clinical diagnosis by laboratory methods, 9th edition, Philadelphia, 355 (1942).
9. Young, E. G., MacPherson, C. C., Wentworth, H. P., and Hawkins, W. W., *J. Biol. Chem.*, **152**, 245 (1944).
10. Peters, J. H., *J. Biol. Chem.*, **146**, 179 (1942).
11. Lu, G. D., *Biochem. J.*, **33**, 249 (1939).
12. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 211 (1914).
13. Benedict, S. R., and Franke, E., *J. Biol. Chem.*, **52**, 387 (1922).
14. Young, E. G., and Conway, C. F., *J. Biol. Chem.*, **142**, 839 (1942).
15. Folin, A., *Z. physiol. Chem.*, **41**, 223 (1904).
16. Collier, H. B., *Canad. Med. Assn. J.*, **50**, 550 (1944).

THE COLORIMETRIC ESTIMATION OF PROTEINS IN
VARIOUS BODY FLUIDS*

By ANTHONY A. ALBANESE, VIRGINIA IRBY, AND BARBARA SAUR

(*From the Children's Medical Service of Bellevue Hospital and the Department of
Pediatrics, New York University College of Medicine, New York*)

In undertaking a study of infantile nephrosis, we were confronted with
the problem of having to determine the total protein content and albumin-
globulin ratios of numerous specimens of blood, urine, and abdominal fluid.
The commonly employed colorimetric (1) and nephelometric (2) methods
yielded results in our hands which did not compare favorably with those of
the time-consuming micro-Kjeldahl procedure. These circumstances led
us to attempt the determination of the proteins in these body fluids in terms
of arginine which can be determined directly in whole proteins by means of
the Sakaguchi reaction (3). Our experiments disclosed that when alkaline
solutions of the protein fractions, obtained by treatment of the specimens
with trichloroacetic acid or sodium sulfate solutions of appropriate concen-
tration, were submitted to the modified Sakaguchi reaction (4) arginine
values were obtained which when factored gave protein figures closely ap-
proximating those secured by the micro-Kjeldahl technique. The ease
and rapidity of operation, high degree of accuracy, and diminished sample
size required in the new procedure have prompted us to discard the Kjeldahl
method for work of this nature.

Reagents—

Sodium hypochlorite. A 0.06 N solution was prepared as needed from the
commercially available Clorox. The necessary dilution of the stock
product was ascertained iodometrically as follows: To 1 cc. of Clorox in a
125 cc. Erlenmeyer flask are added 25 cc. of chlorine-free water in which has
been dissolved 1 gm. of KI. The mixture was then titrated with 0.1 N
sodium thiosulfate, 1 cc. of starch indicator being used. The stock product
has been found to be fairly stable for 3 to 4 months if kept in the refrigerator
in a brown bottle.

Sodium hydroxide. 10 per cent solution.

Urea. 20 per cent solution.

α-Naphthol. 100 mg. of the resublimed product are dissolved in 100 cc.
of 95 per cent ethanol. The solution is kept in a brown bottle and stored
in the refrigerator.

231

Arginine standard. 12.05 mg. of $l(+)$-arginine hydrochloride, Merck (26.6 per cent N found), are weighed accurately and dissolved in 100 cc. of a saturated aqueous solution of benzoic acid. 1 cc. of this solution is equivalent to 100 γ of the free base. This solution is stored in the refrigerator.

Ether. Reagent grade can be used as obtained.

Trichloroacetic acid. 5, 10, and 30 per cent solutions are required.

Sodium sulfate, anhydrous. 22 and 44 per cent solutions are employed and are stored in a 37° incubator.

Analytical Procedure

Blood. Total Proteins—0.5 cc. of plasma was transferred to 10 cc. of 10 per cent trichloroacetic acid in a 15 cc. centrifuge conical tube and after standing for 10 minutes at room temperature centrifuged at 3000 R.P.M. for 10 minutes. The supernatant fluid was decanted into a 100 cc. Kjeldahl flask and treated in the customary manner for the determination of non-protein nitrogen by the micro-Kjeldahl method. The precipitated protein was washed by resuspension in 5 cc. of 5 per cent trichloroacetic acid, centrifugation, and decantation of the supernatant fluid. The washed protein was dissolved in 10 cc. of 10 per cent NaOH and 1 cc. aliquots were removed for the arginine determination.

Globulin—0.5 cc. of plasma was added with mixing to 10 cc. of 22 per cent sodium sulfate in a 50 cc. round bottom, small neck centrifuge tube and incubated at 37° for 3 hours or more. At the end of the incubation period, 3 cc. of ether were added and then the heterogeneous mixture was shaken vigorously and centrifuged for 15 minutes at 3000 R.P.M. The protein which collected at the ether-water interfaces was separated from both liquid layers by careful decantation. The protein cake was washed by resuspension in 10 cc. of 22 per cent sodium sulfate and 3 cc. of ether, centrifugation, and decantation of the liquids as described before. The remaining protein disk was dissolved in 10 cc. of 10 per cent NaOH and 1 cc. aliquots removed for the arginine determination. Gentle heating of the mixture in a hot water bath drives off the residual ether and thereby greatly facilitates solution of the protein.

When only limited amounts of the sample are available, the total protein and globulin determinations can be performed on 0.1 and 0.2 cc. of blood plasma in 15 cc. centrifuge tubes respectively by a proportionate decrease of all the reagents. Owing to the low non-protein nitrogen content of the trichloroacetic acid filtrates, this component is determined as follows in the micro adaptation of the method. After digestion of the supernatant fluid has been completed, 5 cc. of 0.01 N ammonium sulfate are added to the sample in order to increase the quantity of N to the range of maximum sensitivity of the micro-Kjeldahl determination. An identical quantity of

ammonium sulfate is in a blank control determination. Distillation and subsequent boric acid titration of the distilled ammonia are carried out according to the procedure of Meeker and Wagner (5). The non-protein N of the sample is then estimated by the difference between the plasma sample and $(NH_4)_2SO_4$ blank readings.

Urine and Ascitic Fluid. Total Proteins—24 hour specimens of urine from nephrotic patients were collected in 2 liter bottles containing 3 cc. of 10 per cent alcoholic thymol. The abdominal fluid was derived by paracentesis and like the urine preserved with alcoholic thymol. To 5 cc. of urine or ascitic fluid in 15 cc. centrifuge tubes were added 2 cc. of 30 per cent trichloroacetic acid with vigorous mixing. After 10 minutes, the tubes were centrifuged for 10 minutes at 3000 R.P.M. The supernatant fluids were discarded by cautious decantation and the precipitated proteins washed by resuspension in 5 cc. of 5 per cent trichloroacetic acid and another centrifugation. The protein fractions were dissolved in 5 cc. of 10 per cent NaOH and 1 cc. aliquots removed for the arginine determination. In the critical stages of the disease, both of these body fluids may contain large quantities of proteins and, in order to bring the colorimetric reading within the proper range, it becomes necessary to make a further dilution (usually 1:5) of the protein solution with 10 per cent NaOH.

Globulin—10 cc. of urine or abdominal fluid were incubated for 3 hours or more at 37° with 10 cc. of 44 per cent sodium sulfate contained in a 50 cc. round bottom, narrow neck centrifuge tube. At the end of this time, 3 cc. of ether were added to each tube and mixed with vigorous shaking. The globulin component was separated by centrifugation and decantation and then washed by a repetition of these steps after resuspension in 10 cc. of 22 per cent sodium sulfate and 3 cc. of ether. The resulting globulin fraction was dissolved in 10 cc. of 10 per cent NaOH.

Colorimetric Determination of Arginine in Whole Proteins—To the 1 cc. aliquots of the alkaline solutions of the proteins obtained from the body fluids are added 5 cc. of water and 1 cc. of α-naphthol reagent with vigorous mixing. After 5 minutes, add 1 cc. of diluted Clorox and exactly 1 minute later add 2 cc. of 20 per cent urea solution. The reaction mixture is allowed to stand for 5 minutes before reading in the Klett-Summerson colorimeter with Filter S-54. A 1 cc. aliquot of the standard and reagent blank is run in parallel with the determination.

Calculations—After the colorimeter is set at 0 for the reagent blank, the arginine present in the protein samples is estimated by comparison with the value of the arginine standard. The protein equivalent is obtained by multiplying the arginine values by 19.2, a factor derived from the observed mean arginine content (5.2 per cent) of albumin and globulin of these body fluids (6). The albumin content is estimated by difference of the total protein and globulin figures.

Results

A linear relationship of color intensity to the amount of protein in the sample under the reaction conditions described is indicated by tests performed on varying quantities of an alkaline solution of a purified urinary globulin, prepared in this laboratory by repeated recrystallization and electrodialysis, which on analysis contained 12.97 per cent N, 0.49 per cent ash, and 5.08 per cent moisture (Fig. 1). This observation recommends the color reaction as performed for analytical purposes. It is also obvious from this that solutions of pure globulin or albumin may be used as standards, but, since these are hard to prepare and difficult to keep, we have therefore preferred to employ an arginine standard.
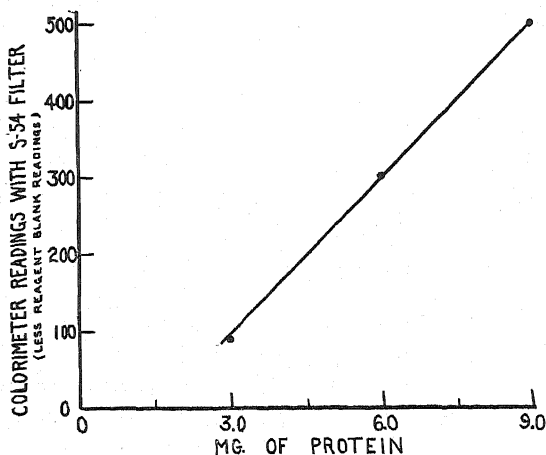
Fig. 1. Relation of color intensity of the Sakaguchi reaction to the amount of purified urinary globulin. Each area represents the average value of five or more determinations performed at 20–25°.

Typical results obtained by submitting aliquots of the same protein solutions derived from the fractionation of various blood, urine, and ascitic fluid samples to the colorimetric and micro-Kjeldahl methods are shown in Table I. The close agreement of the figures obtained by the two methods affirms the use of the factor 19.2, which was based on the values in the literature, for arginine in albumin and globulin of the blood, nephrotic urine, and abdominal fluids. Conversely, these data suggest that the arginine contents of these proteins are quite similar, a deduction supported by the results of amino acid analysis of the highly purified protein preparations which we have isolated from these body fluids and which will be reported subsequently.

The limited quantity of blood which is often obtainable from infants

under study led us to attempt the estimation of the proteins on smaller amounts of plasma than are usually employed. These experiments showed that a minimum of 0.1 and 0.2 cc. of plasma was required for the estimation of the total proteins and globulins with an accuracy comparable to that of 0.5 cc. runs. The accord of data obtained on samples which were submitted

TABLE I

Comparison of Results of Protein Analysis of Various Body Fluids Obtained by Micro-Kjeldahl and Arginine Colorimetric Methods

Specimens		Micro-Kjeldahl method Protein = N × 6.25				Arginine method Protein = arginine × 19.2				(a) − (b)
		Total protein	Albumin	Globulin	A:G ratio (a)	Total protein	Albumin	Globulin	A:G ratio (b)	
		gm. per cent	gm. per cent	gm. per cent		gm. per cent	gm. per cent	gm. per cent		
Normal adult	Al	6.55	4.64	1.91	2.43	6.60	4.68	1.92	2.44	−0.01
plasmas	Ha	5.86	4.07	1.79	2.27	5.90	4.06	1.84	2.21	+0.06
	Ag	6.50	4.27	2.24	1.91	6.52	4.24	2.28	1.86	+0.05
	Al	Micro adaptation*				6.48	4.58	1.90	2.41	+0.02
	Ha					5.82	3.98	1.84	2.16	+0.11
	Ag					6.50	4.24	2.26	1.88	+0.08
Plasma from nephrotic children	Do, female, 4 yrs.	4.42	1.21	3.21	0.38	4.53	1.24	3.29	0.38	0.00
	To, male, 2 yrs.	4.29	1.74	2.55	0.68	4.35	1.70	2.65	0.64	+0.04
	Sa, female, 3 yrs.	3.69	1.07	2.62	0.40	3.70	0.99	2.71	0.36	+0.04
24 hr. urine from nephrotic children	Do, 1300 cc.	1.57	0.96	0.61	1.57	1.59	0.97	0.62	1.56	+0.01
	To, 1600 "	0.61	0.42	0.19	2.21	0.61	0.42	0.19	2.21	0.00
	Sa, 1300 "	0.88	0.65	0.23	2.83	0.86	0.63	0.23	2.78	+0.05
Ascitic fluid from nephrotic children	Do, 4600 "	0.10	0.05	0.05	1.00	0.11	0.06	0.05	1.20	−0.20
	To, 6000 "	0.24	0.11	0.13	0.85	0.24	0.12	0.12	1.00	−0.15
	Sa, 3800 "	0.18	0.11	0.07	1.57	0.18	0.11	0.07	1.57	0.00

* 0.1 and 0.2 cc. aliquots of the above plasma were used for total protein and globulin determinations, respectively.

to the macro- and microcolorimetric and micro-Kjeldahl techniques indicates the analytical suitability of the micro modification.

A statistical examination of the results on the various protein fractions reveals that the average difference between the albumin-globulin ratios derived by the arginine and micro-Kjeldahl methods is about 2 per cent.

Comments

Our experiences with the two colorimetric procedures employing the phenol reagent or the sulfosalicylic acid reagent disclosed that the ease and

rapidity of operation embodied in these methods entailed too great a sacrifice in accuracy to warrant their adoption except for purely comparative purposes. We, too, as have others before us (7), found the results of blood protein analysis by these techniques to show a variation of 10 per cent or more from the values obtained by the micro-Kjeldahl method. We have found that the differences in data secured by these colorimetric techniques and the micro-Kjeldahl method were as great as 50 per cent for the proteins of urine and ascitic fluid. These discrepancies are to be contrasted with the 2 per cent variation for all the reported protein fractions between the arginine and micro-Kjeldahl techniques.

The cause of the greater accuracy of the arginine method compared with the other methods arises from the apparently uniform arginine content of these proteins as contrasted to their variability in dispersion characteristics and tyrosine content. Our inability to obtain suitable data with the sulfosalicylic acid procedure evidently arose from differences in dispersion behavior of albumin and globulin, since we later observed that widely divergent optical density values were given by solutions containing identical amounts of purified albumin and globulin. The inherent errors of the tyrosine procedure can be readily realized when it is noted that the equivalents reported range from 12.9 to 27.5 mg. of albumin and 15.8 mg. of globulin per mg. of tyrosine (8). It is apparent therefore that in terms of heterogeneous proteins of variable composition the results can only be considered valuable for relative comparison. Other variations may be caused by the presence in proteins of factors other than tyrosine which will give colors with the phenol reagent (9).

SUMMARY

A colorimetric method for the estimation of total proteins and albumin-globulin ratios of plasma, nephrotic urine, and abdominal fluid by the Sakaguchi reaction has been described which has an accuracy comparable to the micro-Kjeldahl method. The high sensitivity of the test permits a determination of these protein components on 0.3 cc. of human plasma and recommends the procedure for use in pediatric studies.

BIBLIOGRAPHY

1. Greenberg, D. M., *J. Biol. Chem.*, **82**, 545 (1929).
2. Folin, O., and Denis, W., *J. Biol. Chem.*, **18**, 273 (1914). Denis, W., and Ayer, J. B., *Arch. Int. Med.*, **26**, 436 (1920).
3. Sakaguchi, S., *J. Biochem.*, Japan, **5**, 133 (1925).

4. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **159**, 185 (1945).
5. Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem.*, *Anal. Ed.*, **5**, 396 (1933).
6. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield, 58–60 (1945).
7. Walker, B. S., and Bakst, H. J., *J. Lab. and Clin. Med.*, **20**, 312 (1934).
8. Andersch, M., and Gibson, R. B., *J. Lab. and Clin. Med.*, **18**, 816 (1933). Hewitt, L. F., *Brit. J. Exp. Path.*, **8**, 84 (1927). Riegler, E., *Z. anal. Chem.*, **53**, 242 (1914).
9. Hanke, M. T., *J. Biol. Chem.*, **79**, 587 (1928).

THE CONVERSION OF CITRULLINE TO ARGININE
(TRANSIMINATION) BY TISSUE SLICES
AND HOMOGENATES*

By PHILIP P. COHEN AND MIKA HAYANO

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

The conversion of citrulline to arginine by a *transimination* reaction involving glutamic acid and metabolically related compounds has been reported to take place in kidney slices by Borsook and Dubnoff (1). These authors found that liver slices did not catalyze the reaction and further that the breakdown of cell structure of kidney tissue, such as by homogenization, resulted in a loss of activity.

In the course of an investigation on the mechanism of urea synthesis in liver, some evidence for the transimination reaction was obtained in homogenates fortified with adenosine triphosphate (ATP), magnesium ions, and cytochrome *c*. These findings, which are reported in a separate publication (2), led to a study of the transimination reaction in kidney and other tissue homogenates. Experiments reported in this paper deal with (1) the conditions required for demonstrating the transimination reaction in tissue homogenates and (2) the relative rates of this reaction in slices and homogenates of kidney and liver.

EXPERIMENTAL

Preparation of Tissues

Slices—Livers and kidneys of adult white rats were removed shortly after the animals had been decapitated, bled, and chilled on ice, and the slices were cut immediately thereafter. For liver studies, three slices, totaling approximately 12 to 15 mg. in dry weight, were used in each flask. In kidney studies, two slices, approximately 7 to 10 mg. in dry weight, were used.

Homogenates—Tissues were removed from adult rats treated in the same manner as described above, and chilled on ice before being cut into small pieces to facilitate homogenization. Homogenization was carried out in the apparatus of Potter and Elvehjem (3). The cold homogenate, after straining through four layers of cheese-cloth, was added to Warburg flasks containing cold medium and substrates. All operations were carried out in a 1° cold room to insure maintenance of low temperatures. The homogenate

239

which was prepared with 2 volumes of buffer (0.0128 M sodium phosphate, pH 7.4, 0.123 M sodium chloride, 0.005 M potassium chloride, 0.0033 M magnesium sulfate) was diluted to the final tissue concentration desired. Liver homogenates, representing 10 to 15 per cent wet weight, were found to contain 2 to 3 mg. of N per ml.; 20 to 25 per cent kidney homogenate contained 4 to 5 mg. of N per ml. Aliquots of the homogenates were taken for analysis of the total nitrogen, on which basis the results are expressed.

A representative homogenate of liver tissue was examined for cytolysis. Macroscopically, it was an opaque homogeneous preparation containing no visibly large particles. A portion of the homogenate was centrifuged and the centrifugate, which represented 10 to 15 per cent of the total volume, was fixed with 10 per cent formaldehyde, embedded in paraffin, sectioned, and stained. Histological examination revealed an occasional clump of cells. It was estimated that about 5 per cent of the homogenate consisted of unruptured cells.

Potter (4) has suggested that the preparation of tissue homogenate in water increases the degree of cytolysis. When this was done and the water homogenate was compared with a buffer homogenate, it was found that the water preparation had 75 per cent of the activity of the buffer homogenate for liver and about 85 per cent for kidney. It is possible that the lower activity of the water homogenates was due to a delay in restoring the isotonicity of the medium.

Incubation

All incubations were carried out in Warburg flasks at 38° for a period of 40 minutes, unless otherwise specified. All flasks were charged with pure tank oxygen just prior to introduction into the bath. For anaerobic experiments tank nitrogen was used with an oxygen-carbon dioxide-absorbing mixture of Van Slyke (5) in the center wells of the flasks.

Substrates and Cofactors

Tissue Slice Experiments—Each flask contained the following components in the main compartment: *dl*-citrulline, glutamic acid or other imino group donor, and tissue slices in a total volume of 3.5 ml. made up with buffer, pH 7.4 (see "Homogenates" under "Preparation of tissues"). At the end of the incubation period, the slices were removed, rinsed in water, and placed in tared cups for drying and weighing. The contents of the flasks were then analyzed for either urea or arginine or both, as described under "Analytical methods."

Homogenate Experiments—Incubation mixtures consisted of *dl*-citrulline, glutamic acid, ATP, cytochrome *c*, homogenate and buffer, pH 7.4, to make a final volume of 3.0 or 3.5 ml., as indicated. In most experiments with

liver homogenates, 3 to 10 units of purified calf liver arginase were added to the incubation mixture to insure complete conversion of arginine to urea. However, control experiments indicated no need for the arginase addition. At the end of the incubation period, the contents of the flasks were analyzed for either urea or arginine or both. Liver incubation mixtures were always analyzed for urea, and arginine when specified. On the assumption that the urea formed by liver preparations was derived from a quantitative hydrolysis of the arginine formed, the results are presented as mg. of arginine formed. Kidney incubation mixtures were always analyzed for arginine, and occasionally for urea, as indicated.

Preparations Used—The *dl*-citrulline used throughout this study was obtained from the Amino Acid Manufactures, University of California, Los Angeles.

l(+)-glutamic acid was a commercial preparation.

ATP was prepared from rabbit muscle following magnesium and barbiturate anesthesia (6). This preparation was 79 per cent pure on the basis of its easily hydrolyzed phosphorus (7 minutes at 100° in 1 N HCl).

Cytochrome *c* was prepared by the method of Keilin and Hartree (7).

l(+)-Ornithine hydrochloride was prepared according to Hunter (8).

Glutamine was obtained from Dr. H. B. Vickery, and cozymase (80 per cent pure) from Dr. G. A. LePage. The authors are indebted to these investigators for these generous gifts.

α-Ketoglutaric acid was prepared by synthesis from ethyl oxalate and ethyl succinate.

Succinamic acid was prepared by the hydrolysis of succinimide.

Arginase was prepared by the method of Hunter and Downs (9).

Analytical Methods

Determination of Urea—Urea was determined by the method of Krebs and Henseleit (10), with a 1 per cent solution of purified urease (The Arlington Chemical Company). Solutions obtained from slices were brought to pH 5.0 with 0.3 ml. of 3 M acetate buffer, and the urea content determined manometrically. Solutions from homogenates were centrifuged after the addition of the acetate buffer to remove the precipitated protein, and an aliquot of the clear supernatant was taken for the urea determination.

Determination of Arginine—All solutions with or without prior urease treatment were treated with 1 ml. of 10 per cent trichloroacetic acid, centrifuged, and an aliquot taken from the clear supernatant. The method employed was essentially that of Dubnoff (11). Samples for colorimetric analysis were prepared as follows: To a 1 ml. aliquot of the deproteinized incubation mixture were added 9 ml. of distilled water and 1 ml.

of the naphthol-urea reagent, followed by 0.5 ml. of sodium hypobromite solution. For the blank, 10 ml. of distilled water plus the reagents were used.

Results

Effect of ATP—The requirements of ATP by liver and kidney homogenates are shown in Fig. 1. In the absence of ATP only a negligible amount of arginine was formed. This was particularly noticeable in studies with liver, in which almost a maximum activity was seen at one-half the ATP concentration $(8 \times 10^{-4}$ M$)$ required for a maximum response with kidney homogenate. However, even with increased amounts of ATP
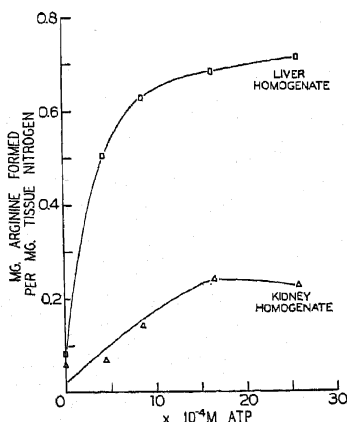
FIG. 1. Effect of adenosine triphosphate concentration on transimination by liver and kidney homogenates. Tissue concentration per flask: liver 1.586 mg. of N; kidney 1.572 mg. of N. Substrate concentrations are the same as those given under Fig. 2 for liver homogenate.

the synthesis by kidney of arginine did not at any time exceed 40 per cent of the activity of liver.

Effect of Magnesium Ions—When increasing concentrations of magnesium ions were used with liver homogenate, a maximum activity was noted at about 3×10^{-3} M concentration (Table I). Stoner and Green (12) have reported that magnesium ions inhibit the deamination and dephosphorylation of ATP. This in all probability is the basis for the activation noted in the present experiments. Magnesium ions in optimum concentration were used throughout the experiments. The effect of magnesium ions on kidney homogenate was not investigated.

Effect of Cytochrome c—Cytochrome c was included throughout the study, although evidence for its requirement has not been consistent. In experi-

ments where stimulation has been noted, this has been as high as 100 per cent. Borsook and Dubnoff (1) have indicated that cytochrome c plays a rôle in the oxidation of the hypothetical citrulline-glutamic acid intermediate.

Comparison of Kidney Slices and Homogenate—Fig. 2 shows the relative rates of arginine formation by kidney slices and homogenate. The data obtained with kidney slices confirm Borsook and Dubnoff's findings (1). These investigators found approximately 0.027 mg. of arginine produced per mg. of dry weight of tissue per hour, or, on the basis of 12.63 per cent nitrogen (determined experimentally) in dry kidney tissue, 0.216 mg. of arginine per mg. of tissue nitrogen per hour. In the present experiments, values of the order of 0.265 mg. of arginine were found at the end of 1 hour incubation. Attempts to demonstrate arginine formation in kidney ho-

TABLE I

Activation of Liver Homogenate by Magnesium Ions

Volume 3 ml.; tissue concentration per flask 0.986 mg. of N. Substrate and cofactor final concentration as follows: dl-citrulline 6.7×10^{-3} M, glutamic acid 6.7×10^{-3} M, cytochrome c 4.75×10^{-5} M, adenosine triphosphate 1.0×10^{-3} M; activated arginase 3 units. The changes in magnesium ion concentrations are balanced by sodium ions.

Magnesium ion concentration	Arginine formed per mg. tissue N
	mg.
3.33×10^{-4} M	0.1113
1.33×10^{-3} "	0.1305
2.67×10^{-3} "	0.1780
6.67×10^{-2} "	0.0673
1.00×10^{-1} "	0.0708

mogenate brought to light a system only about 40 per cent as active as that in intact slices. This system, however, showed a greater specificity as to the source of the imino group donator, namely glutamic acid, and a dependence on the presence of ATP.

Comparison of Liver Slices and Homogenate—In contrast to kidney slices and homogenate, liver homogenate was found to contain an unusually active system having about 80 per cent greater activity than kidney slices at 1 hour, and a 500 per cent greater activity than kidney homogenate and liver slices. The requirements of the liver homogenate for the necessary components, ATP (Fig. 1), citrulline (Fig. 3), and glutamic acid (Fig. 4), were of much smaller magnitude than that required by kidney homogenate. Liver slices, on the other hand, showed a very low activity.

Comparison of Liver and Kidney Preparations. Oxygen—The require-

ment of the transimination system for oxygen as first shown with kidney slices by Borsook and Dubnoff (1) has been confirmed in the present studies. Under an anaerobic atmosphere, neither slices nor homogenates of both liver and kidney synthesized arginine.
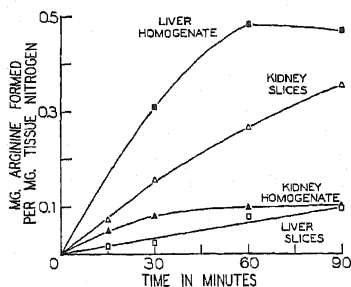
FIG. 2. Comparison of the rates of transimination by different tissue preparations. Tissue concentration per flask: liver homogenate 1.46 mg. of N; liver slices 15 to 20 mg. dry weight; kidney homogenate 1.38 mg. of N; kidney slices 7 to 9 mg. dry weight. Substrate and cofactor final concentration as follows: liver homogenate and slices, *dl*-citrulline and glutamic acid, each at 5.72×10^{-3} M, adenosine triphosphate 8.57×10^{-4} M, cytochrome *c* 4.72×10^{-6} M; following variations employed with kidney slices and homogenate: homogenate, adenosine triphosphate 1.14×10^{-3} M; slices, glutamic acid 8.57×10^{-3} M.
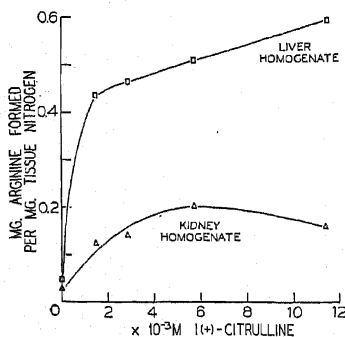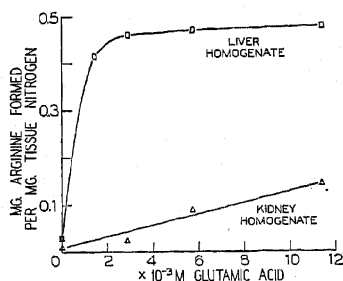

FIG. 3. Effect of citrulline concentration on transimination by liver and kidney homogenates. Tissue concentration per flask: liver 1.461 mg. of N, kidney 1.572 mg. of N. Substrate concentration is the same as that given under Fig. 2 for liver homogenate. *dl*-Citrulline was used as the substrate in twice the concentration given as the *l*(+) form on the abscissa.

Citrulline—The relative utilization of citrulline by both liver and kidney homogenates is shown in Fig. 3. Without citrulline no appreciable amounts of arginine were formed. The optimum citrulline concentration for kidney was found to be approximately 6×10^{-3} M. In the case of liver a rapid

rise in arginine formation resulted from an increase in citrulline concentration to approximately 1.5×10^{-3} M; beyond this point the synthesis of arginine was less strikingly influenced by the citrulline concentration. At optimum concentrations of citrulline for both kidney and liver homogenates, the former had only 40 per cent of the activity of the latter.

Transimination with Compounds Similar to Glutamic Acid—Borsook and Dubnoff (1) found that kidney slices showed a considerable utilization of compounds metabolically related to glutamic acid. Glutamine and aspartic acid were equally as effective as glutamic acid, and asparagine, glutathione, lysine, proline, and ornithine about half as effective. It was found in the present study with homogenates that substrate specificity was more limited (Table II). Thus with both homogenates glutamine was the only

TABLE II

Percentage Transimination with Compounds Related to Glutamic Acid in Liver and Kidney Homogenates

Glutamic acid taken as 100 per cent.

Substance in addition to citrulline	Kidney, relative rate	Liver, relative rate
Glutamic acid	100.0	100.0
Glutamine	84.5	71.9
Aspartic acid	15.8	22.8
Asparagine	23.2	19.0
α-Ketoglutaric acid + ammonium chloride	19.4	5.8
Ammonium chloride	0.0	7.3
Ornithine	2.2	0.0
Lysine	19.1	
Succinamic acid		27.5
Lactic acid + ammonium chloride		2.5

compound other than glutamic acid showing an appreciable effect. The activity of glutamine was to be expected in view of the active glutaminase system in both tissues. Aspartic acid and asparagine were less than one-fourth as active as glutamic acid. The failure in the utilization of ammonium chloride by kidney homogenate is in agreement with the work on slices. In contrast to liver slices, liver homogenate failed to utilize ammonium chloride (7.3 per cent as effective as glutamic acid), which appears to be metabolized more rapidly than glutamic acid in the synthesis of urea by slices (2). α-Ketoglutaric acid plus ammonium chloride showed only 20 per cent the activity of glutamic acid with kidney homogenates. Borsook and Dubnoff found a 46 to 80 per cent activity with this combination with kidney slices. Liver homogenate was less efficient than kidney with these substrates. Ornithine, which was 42 per cent as active as glutamic acid in

kidney slices, yielded no arginine with either kidney or liver homogenates. Apparently the mechanism for converting ornithine and other potential imino group donators to glutamic acid (13) is lost when kidney tissue is homogenized. Succinamic acid with liver homogenate showed a relative effectiveness of 27.5 per cent. The basis for the activity of this compound

FIG. 4. Effect of glutamic acid concentration on transimination by liver and kidney homogenates. Tissue concentration per flask: liver 1.461 mg. of N; kidney 2.22 mg. of N. Substrate concentrations are the same as those given under Fig. 2 for liver homogenate.
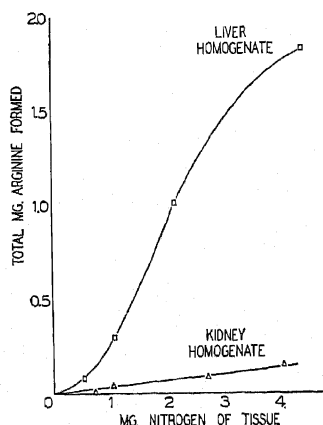
FIG. 5. Effect of tissue concentration on transimination by liver and kidney homogenates. The substrate concentrations are the same as those given under Fig. 2 for liver homogenate.

other than by metabolic conversion to an α-amino compound is obscure. Lactic acid and ammonium chloride, reported by Krebs and Henseleit (10) to increase the amount of urea (or arginine) produced by liver slices over that of ammonium chloride used alone, was ineffective with homogenate.

Glutamic Acid—The requirements for glutamic acid by the two tissue

homogenates are shown in Fig. 4. Increasing the glutamic acid concentration beyond 3×10^{-3} M resulted in no further increase in arginine formation by the liver, while kidney evidently required a concentration greater than 12×10^{-3} M. Even at this concentration, however, the activity of kidney was only one-third of that observed in liver.

Cofactors—The possibility of necessary cofactors was investigated. A boiled aqueous rat liver extract was added to the system without any significant effect. Pyridoxal and pyridoxamine were also tried with negative results. The addition of coenzyme I resulted in some stimulation with low tissue concentrations.

Tissue Concentration—Increasing the amounts of tissue (Fig. 5) brought about a remarkable increase in arginine formation by liver, which was not paralleled by kidney. With a higher level of liver tissue per cup, the blank

TABLE III

Arginine Formation by Different Tissue Homogenates

The substrate and cofactor final concentration are the same as in Table IV.

Tissue	Tissue N per flask	Urea formed per mg. tissue N	Urea equivalents expressed as arginine per mg. tissue N (calculated from column III)	Arginine found per mg. tissue N
(I)	(II)	(III)	(IV)	(V)
	mg.	*microliters*	*mg.*	*mg.*
Liver.............	2.750	60.5	0.471	0.011
Kidney...........	2.686	4.01	0.031	0.091
Heart.............	1.497	0.69	0.005	0.013
Testes............	3.465	−0.04	0.0	0.0
Brain.............	1.810	−0.41	0.0	0.017

values rose proportionately. At a tissue level of 1 to 1.5 mg. of N per flask, however, blank values were practically zero, and consequently this level was used throughout the study. With kidney homogenates, on the other hand, increasing the tissue concentration resulted in no striking increases in arginine synthesis. Activity at a level of 4 mg. of tissue N per flask was only about 10 per cent that of liver.

Arginine Formation by Various Rat Tissue Homogenates—Data from Table III indicate that liver and kidney homogenates were the only tissues showing any appreciable arginine formation from citrulline and glutamic acid. Both urea and arginine were determined in all instances. The relative arginase activities of different tissues have been reported to be as follows (10): liver 2000, kidney 67, testes and heart 0. It would be expected that any arginine formed by liver would be rapidly and quantitatively

converted to urea.   This was borne out in the present experiment in which it is to be noted that whereas 60.5 microliters of urea (corresponding to 0.471 mg. of arginine) were found, the amount of arginine present was negligible.   With kidney homogenate, on the other hand, arginine accumulated as such, only a small portion being converted to urea.

Effect of Inhibitors—Inhibition by cyanide and arsenite at a final concentration of 0.001 M was reported by Borsook and Dubnoff to be about 95 per cent complete in the case of kidney slices (1).   With liver homogenate cyanide and arsenite inhibited 86 and 99 per cent respectively (Table IV).   Fluoride and iodoacetate have been reported to inhibit the regeneration of ATP and phosphorylation mechanisms (14). The inhibition observed in the present study (fluoride 98 per cent, iodoacetate 74 per cent) might be explainable on this basis.   While inhibition by

TABLE IV

Effect of Inhibitors on Liver Homogenate

Tissue concentration per flask, 1.573 mg. of N.   Substrate and cofactor final concentration as follows: *dl*-citrulline 5.72×10^{-3} M; glutamic acid 5.72×10^{-3} M; cytochrome c 4.75×10^{-6} M; adenosine triphosphate 8.58×10^{-4} M.

Inhibitors	Final concentration (molarity)	Per cent inhibition
Sodium arsenite	0.001	99.3
" fluoride	0.01	98.4
" malonate	0.0057	90.2
Potassium cyanide	0.001	85.8
Sodium iodoacetate	0.01	74.4
" azide	0.001	23.8
Calcium ions	0.001	90.0

malonate at lower concentrations of the order of 0.001 M has been ascribed to inhibition of succinoxidase activity, concentrations of 0.01 M and higher are thought to influence other systems.   The mechanism of malonate inhibition in the transimination system reported here is not apparent. Inhibition of the cytochrome system by azide has been described by Keilin and Hartree (15), who reported its action as being less complete than that with cyanide.   The transimination system in this study showed 24 per cent inhibition with azide, and 86 per cent with cyanide.

In addition to the inhibitors named above, calcium ions were also found to inhibit the system.   This inhibition was not relieved by the addition of 0.001 M magnesium ions over that already present in the buffer nor by an increase in ATP concentration to 1.7×10^{-3} M.

DISCUSSION

It appears from these results that the transimination reaction is in some manner intimately associated with a high energy phosphate donator system. At least the system is active in the presence of ATP when cells are disrupted. This effect is more striking in the case of liver which actually shows a more active transimination system with homogenates than with slices. On the other hand, kidney homogenates, while active, never equaled slices in their transimination activity. This may be accounted for in part by the possibility that the optimum conditions which were established in the case of liver were not optimum when applied to kidney homogenates. Thus it is possible that components other than, or in addition to, those found active in the case of liver are necessary for kidney. In addition, the presence of more active phosphatases in kidney preparations as compared with liver may also be responsible for the lower activity on the basis of more rapid ATP breakdown.

The significance of the transimination reaction in kidney as a mechanism for arginine synthesis has been pointed out by Borsook and Dubnoff (1). As pointed out by Krebs (16), this reaction may account for a significant amount of urea synthesis by transfer of arginine formed by transimination in the kidney to the liver, where it would be broken down to ornithine and urea by arginase.

The most striking finding of the present experiments, however, is that liver homogenates are capable of carrying out the transimination reaction at an extremely rapid rate even exceeding that of kidney slices. The importance of this reaction in urea synthesis by liver is apparent. The details of this reaction in liver are discussed in a separate publication (2).

It would appear from the present study that the transimination reaction is limited chiefly to liver and kidney. Apparently, only the lack of a highly active arginase system in kidney prevents that organ from contributing a significant amount of urea to the total metabolic urea of the body.

SUMMARY

1. The conversion of citrulline to arginine by transimination with glutamic acid in kidney slices and homogenates was studied. The relative activity of the reaction by these preparations was as follows: liver homogenate > kidney slices > kidney homogenate > liver slices.

2. Homogenized tissue preparations of liver and kidney required the following substrates and cofactors: adenosine triphosphate, citrulline, glutamic acid, cytochrome c, magnesium ions, and an oxygen atmosphere. Liver in every case required smaller concentrations of the substrates and

showed a greater activity than kidney homogenate. The significance of these findings is discussed.

3. Aside from glutamic acid, glutamine was the only compound showing any appreciable activity in the transimination system.

4. Brain, testes, and heart homogenates showed no transimination activity.

BIBLIOGRAPHY

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **141**, 717 (1941).
2. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 251 (1946).
3. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
4. Potter, V. R., *J. Biol. Chem.*, **163**, 437 (1946).
5. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 121 (1927).
6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
7. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
8. Hunter, A., *Biochem. J.*, **33**, 27 (1939).
9. Hunter, A., and Downs, C. E., *J. Biol. Chem.*, **155**, 173 (1944).
10. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
11. Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941).
12. Stoner, H. B., and Green, H. N., *Biochem. J.*, **39**, 474 (1945).
13. Krebs, H. A., *Enzymologia*, **7**, 53 (1939).
14. Ostern, P., Baranowski, T., and Terszakowec, J., *Z. physiol. Chem.*, **251**, 258 (1938).
15. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127**, 169 (1939).
16. Krebs, H. A., *Biochem. J.*, **36**, 758 (1942).

UREA SYNTHESIS BY LIVER HOMOGENATES*

By PHILIP P. COHEN and MIKA HAYANO

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison*)

The Krebs-Henseleit theory (1) of urea synthesis (see the modified scheme below) has received support from the later work of Krebs (2, 3), of Gornall and Hunter (4), and from isotope studies (5–8).
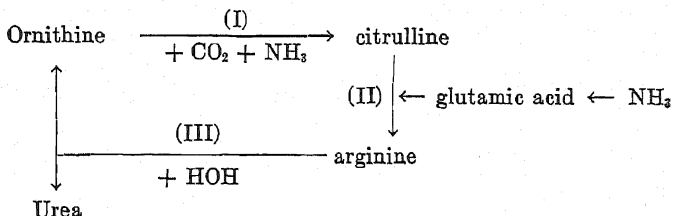
The synthesis of citrulline from ornithine and arginine from citrulline has heretofore been associated only with intact liver cells. In the course of studies on the mechanism of the conversion of citrulline to arginine, a highly active system was found in liver homogenate capable of forming urea in the presence of glutamic acid, adenosine triphosphate (ATP), cytochrome *c*, an oxygen atmosphere, and magnesium ions.

The obvious similarity of the substrates, citrulline and glutamic acid, to that required for the transimination system found by Borsook and Dubnoff (9) in kidney slices led us to a general study of this reaction in rat tissue slices and homogenates (10). The rôle of glutamic acid as the imino group donor to arginine was shown to be very specific, not replaceable by other similar compounds (except glutamine) or by ammonium chloride. In previous work (11), it was reported that liver slices synthesized urea to a limited extent only from citrulline and glutamic acid, more readily from ammonium chloride, and to a considerable extent when both ammonium chloride and glutamic acid were provided. In the present paper, data will be presented which demonstrate in liver the presence of a potent transimination system which in all probability is the mechanism by which citrulline is converted to arginine in the urea cycle.

Procedures

Incubation of Liver Slices and Homogenates—The detailed procedures followed for experiments with liver slices and homogenates have been pre-

251

viously described (10). Minor changes were made in the incubation systems employing slices. Warburg flasks with side arms were used for both incubation and urea determination. Each flask contained glucose, *dl*-citrulline, and Krebs-Ringer-phosphate buffer, pH 7.4, in the main compartment. The side arm contained either ammonium chloride or glutamic acid or both. The final volume in the flasks was either 3.5 or 3.9 ml. as indicated. 20 per cent aqueous potassium hydroxide was used in the center well.

Oxygen Uptake Determinations—For oxygen uptake determinations, the incubation mixtures were prepared as described. All flasks were charged with pure tank oxygen prior to introduction into the bath. 20 per cent aqueous potassium hydroxide was used in the center wells.

Determination of Urea—Urea was determined manometrically with a purified urease preparation by the method of Krebs and Henseleit (1).

Determination of Citrulline—Citrulline was determined in aliquots after urea decomposition with a purified urease prepared according to the method of Archibald and Hamilton (12). After the analysis for urea, the contents of the flasks were deproteinized with 1 ml. of 10 per cent trichloroacetic acid, centrifuged, and an aliquot of the clear supernatant taken for the analysis of citrulline by the colorimetric method of Archibald (13). It was found that these aliquots contained very little chromogenic material other than citrulline, and consequently the Amberlite blank was omitted.

Determination of Glutamic Acid—Glutamic acid was determined manometrically on an aliquot of the incubated sample brought to pH 5.0 with 3 M acetate buffer, with a lyophilized preparation of *Escherichia coli*. The authors are indebted to Dr. R. H. Burris for a generous supply of this preparation.

Determination of α-Ketoglutaric Acid—α-Ketoglutaric acid was determined according to the method of Krebs (14).

Results

Urea Synthesis by Liver Homogenate—The formation of urea from citrulline through arginine by transimination with glutamic acid was shown to occur at an unusually high rate as compared with slices (10). Comparative rates of urea synthesis from glutamic acid, ammonium chloride, or both are shown in Fig. 1. The amount of urea formed in the presence of glutamic acid by homogenate exceeded that formed from other substrate combinations with both homogenates and slices. The maximum amount of urea formed with glutamic acid occurred after 60 minutes. The failure of homogenate after 1 hour may have been due to a depletion of ATP or the coenzymes. With ammonium chloride and glutamic acid, the urea formed in the first 20 to 30 minutes was almost equal to that formed by glutamic

acid alone; however, after this time the rate was not maintained. In all probability, this effect is related to the toxic effect of ammonium ions, which would accumulate during the early incubation period. This system is apparently sensitive not only to increases in ammonium ions but also to α-ketoglutaric acid (Table I).

Urea Synthesis by Liver Slices—Krebs (1) found that urea was formed more rapidly when glutamic acid or lactic acid and ammonium chloride, rather than ammonium chloride alone, was present in the incubation mix-
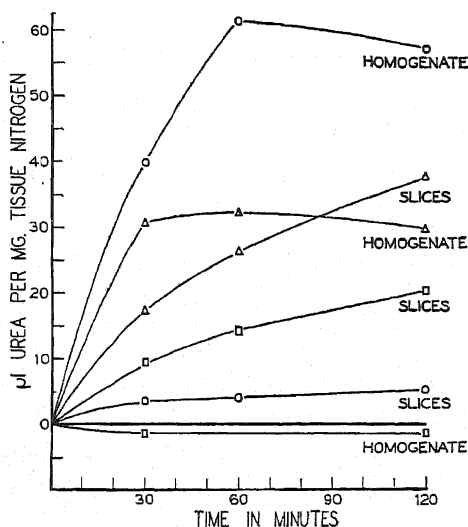
Fig. 1. Relative rates of urea synthesis with different substrates by liver homogenate and slices. Substrate and cofactor final concentration are as follows: Slices, glucose, ammonium chloride, glutamic acid, each at 1.54×10^{-2} M, *dl*-citrulline 3.08×10^{-3} M; tissue dry weights 12 to 17 mg. Homogenates, *dl*-citrulline and glutamic acid, each at 5.72×10^{-3} M, cytochrome *c* 4.72×10^{-6} M, adenosine triphosphate 8.57×10^{-4} M; tissue concentration per flask 1.46 mg. of N. Final flask volumes as follows: slices 3.9 ml., homogenates 3.5 ml. $\triangle$ represents NH_4Cl, and glutamic acid; $\square$ represents NH_4Cl; and $\bigcirc$ glutamic acid.

ture. This observation has been confirmed in the present study as regards glutamic acid (Fig. 1). Urea synthesis from citrulline proceeded more rapidly when both glutamic acid and ammonium chloride were present in the medium. Ammonium chloride alone yielded only about 50 per cent as much urea at the end of a 2 hour incubation period, while glutamic acid alone gave rise to only 15 per cent.

Comparison of Optimum Systems of Slices and Homogenates—On a mg. of tissue N basis with optimum systems of slices and homogenates, the urea

formed by homogenate from glutamic acid and citrulline was more than twice that of the optimum system (citrulline plus glutamic acid plus ammonium chloride) for slices (Fig. 1). It was found that by increasing the tissue concentration in homogenate studies to 2.5 to 3.0 mg. of N per flask the reaction would go to completion within 2 hours. Tissue slices at the rate shown on Fig. 1 would take more than 5 hours.

Comparison of Utilization of Substrates by Homogenates and Slices—Since measurements of the disappearance of glutamic acid in experiments with slices revealed that only 1 per cent disappeared in 1 hour, one-half of which was accounted for as urea, it would indicate that slices are unable to utilize glutamic acid in the system investigated to any appreciable extent. In all probability, the limiting factor is the inability of glutamic acid to diffuse

TABLE I

Inhibition of Urea Synthesis in Homogenates by Ammonium Chloride and α-Ketoglutaric Acid

Volume 3.5 ml. Substrate and cofactor final concentration as follows: Group A, *dl*-citrulline 3.42×10^{-3} M, glutamic acid 1.43×10^{-2} M, adenosine triphosphate (ATP) 1.14×10^{-3} M, cytochrome *c* 4.72×10^{-6} M, tissue concentration 1.087 mg. of N. Group B, *dl*-citrulline 5.72×10^{-3} M, glutamic acid 5.72×10^{-3} M, cytochrome *c* 4.72×10^{-5} M, ATP 8.57×10^{-4} M, tissue concentration 1.482 mg. of N.

Group	Inhibitor added, final molarity	Urea formed per mg. tissue N
		microliters
A. Ammonium chloride	0.0	37.45
	0.00715	35.90
	0.0143	33.30
	0.0286	23.68
B. α-Ketoglutaric acid	0.0	42.40
	0.01142	13.31

through the liver cell wall at a sufficiently rapid rate. In order to evaluate this possibility, experiments were set up in which the oxygen uptake of liver slices and homogenates were compared in the presence of the same substrates in concentrations found to be optimum for urea synthesis. Liver has been known to contain the most active glutamic acid dehydrogenase system of all the tissues studied (15). The relative rates of oxidation of glutamic acid and related substrates in experiments with slices and homogenates would be expected to be a measure of the diffusibility of the glutamic acid through the cell wall of the slices. Homogenates were fortified with all the known necessary cofactors. Since the oxygen uptakes are expressed on a tissue nitrogen basis, it would be assumed that the potential enzyme activity of the two preparations on this basis would be equal.

Any differences in the activity of the two systems would, therefore, in all probability be due to either the limiting rate of diffusion into the liver slices or the inadequate fortification of the homogenate. As seen from Figs. 2 and 3, the rate of oxygen consumption of the homogenate systems is of the order of 6 times greater in the case of glutamic acid than for slices. It should also be noted that the slices had a final glutamic acid concentration 2.5 times greater than that used with homogenates. These results
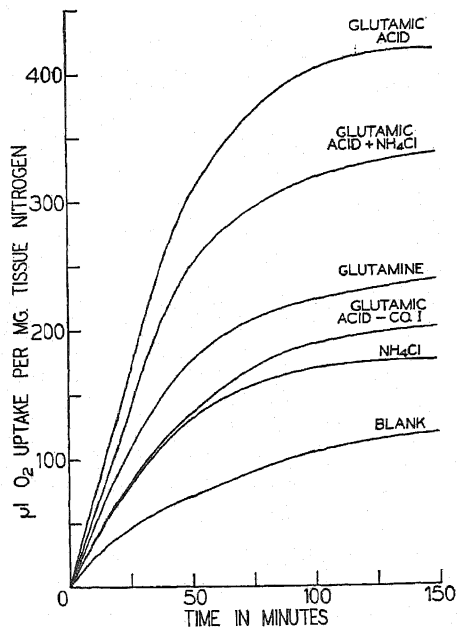
FIG. 2. Relative rates of oxygen uptake with different substrates by liver homogenate. Volume 3.5 ml. Tissue concentration per flask 2.5 mg. of N. Substrate and cofactor final concentration as follows: dl-citrulline, glutamic acid, glutamine and ammonium chloride, each at 5.72×10^{-3} M, adenosine triphosphate 8.58×10^{-4} M, cytochrome c 4.72×10^{-5} M, cozymase (coenzyme I) 8.46×10^{-3} M, nicotinamide 8.2×10^{-3} M.

can mean only that the inability of liver slices to utilize glutamic acid for either oxidation or transimination is due to the relatively poor diffusibility of this compound. The somewhat greater urea-synthesizing activity of the combination of glutamic acid plus ammonia with slices than the additive activities of the two compounds separately may be due to either (1) an effect of the ammonium ion on the diffusibility of glutamic acid into the cell or (2) the probability that ammonia is utilized in Step I by a reaction not involving glutamic acid as an intermediate. The experiments on oxygen

consumption of slices and homogenates do not support the first suggestion but neither do they exclude this possibility. The second suggestion could explain why the combination of glutamic acid plus ammonia, or glutamine, would provide optimum amounts of substrate for Steps I and II. Glutamic acid alone diffuses into the liver slice too slowly to provide an adequate concentration of either ammonia or glutamic acid for Steps I and II respectively, and thus yields little urea. On the other hand, ammonium chloride would be expected to provide an adequate concentration of ammonium ions for synthesis of intermediates for Step I and also to give rise to glutamic acid through the glutamic dehydrogenase system. Findings of experiments in which isotopic ammonia was used lend support to the latter possibility (8). The high activity of glutamine in urea synthesis with
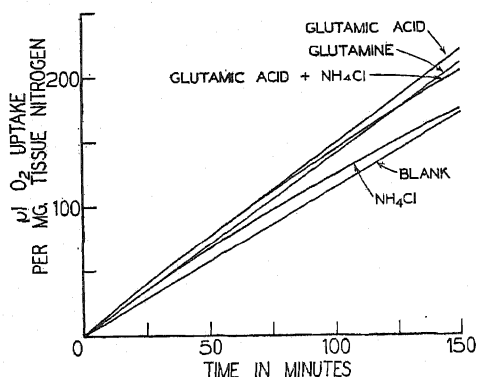
Fig. 3. Relative rates of oxygen uptake with different substrates by liver slices. Volume 3.5 ml. Tissue dry weights 21 to 24 mg. Substrate and cofactor final concentration as follows: glucose, glutamic acid, glutamine and ammonium chloride 1.43×10^{-2} M, dl-citrulline 2.86×10^{-3} M.

slices is in all probability due to its hydrolysis to glutamic acid and ammonia, the optimum substrate combination for slices. The fact that glutamine is more active in liver slices from fasted animals than ammonium chloride suggests that fasted liver lacks the necessary substrates for the sufficiently rapid synthesis of glutamic acid from α-ketoglutaric acid and added ammonia, and thus Step II of the cycle is the limiting reaction.

The failure of ammonia to react in the homogenate system is in all probability due to the fact that conditions for glutamic acid synthesis from ammonia were not optimum. Although the oxygen consumption experiments (Figs. 2 and 3) show a somewhat lower oxygen uptake in the case of glutamic acid plus ammonia in homogenate as compared with glutamic acid alone, this may be due to the inhibitory effect of ammonium ions (Table I) on the glutamic dehydrogenase system (15).

Balance Studies—Additional evidence that the formation of arginine from citrulline by homogenates involves the transimination reaction of Borsook and Dubnoff (9) is that the glutamic acid and citrulline disappearance can be accounted for mole for mole in the formation of urea. Thus, the ratio obtained of urea formed to glutamic acid and citrulline disappearance was 1:1.21:0.93 (Table II). The high value for glutamic acid is undoubtedly due to the fact that it is oxidized readily in the homogenate system (Fig. 2). The failure to find any accumulation of α-ketoglutaric acid in this system may be due either to its ready oxidation or to the possibility that this compound is not an end-product of the reaction.

Conversion of Ornithine to Citrulline by Homogenate—A system including ornithine plus glutamic acid or ammonium chloride, or both, in the presence of a carbon dioxide-bicarbonate buffer was investigated as to its activity in catalyzing Step I (see the accompanying scheme) of the urea cycle. No urea synthesis with this system occurred indicating that the factors required for Step II were inadequate for Step I.

TABLE II

Balance Study of Components of Transimination Reaction

Volume 3.6 ml. Tissue concentration per flask 1.822 mg. of N. Substrate and cofactor final concentration as follows: *dl*-citrulline 4.95×10^{-3} M, glutamic acid 4.91×10^{-3} M, cytochrome *c* 4.62×10^{-6} M, adenosine triphosphate 1.11×10^{-3} M.

Components	Formation or disappearance of substrate and end-products
	micromoles
Glutamic acid	−4.97
Citrulline	−3.83
Urea	+4.12
α-Ketoglutaric acid	0.00

DISCUSSION

With the exception of the hydrolytic conversion of arginine to urea and ornithine by arginase, our knowledge of the enzymatic steps in the urea cycle is obscure. Since the over-all process is an endergonic one, the synthetic steps in the cycle must be coupled with energy-yielding systems. The findings presented in this paper offer strong evidence in support of the coupling of one step (Step II) in this cycle with adenosine triphosphate, and thus establish at least one pathway for coupling the endergonic urea cycle with exergonic oxidation-reduction systems.

The inability of the system catalyzing Step II to convert ornithine to citrulline (Step I) under the conditions employed indicates that Step I is a more complex reaction and probably involves at least two independent

reactions, as previously suggested by Srb and Horowitz (16) and Krebs (17). A study of this phase of the cycle is at present under investigation.

The mechanism of the transimination reaction has been considered in some detail by Borsook and Dubnoff (9) who were led by the evidence available to postulate an intermediate addition compound of citrulline plus glutamic acid with the subsequent dehydrogenation of the intermediate and the formation of arginine and α-ketoglutaric acid (or some related compound). The data from the present investigation bear directly on the mechanism of the reaction only in so far as they establish the fact that 1 mole of arginine (or urea) is formed for every mole of glutamic acid and citrulline which disappears, and further that in confirmation of Borsook and Dubnoff's findings some suitable hydrogen acceptor must be present. The high activity of the homogenate system, however, provides a more satisfactory system for studying the transimination reaction in detail, and this is at present being done.

Finally, it should be pointed out that from the findings of the present paper it would appear that glutamic acid is an obligatory intermediate in the conversion of citrulline to arginine (Step II) in the urea cycle. This may resolve the somewhat different views held on certain aspects of the urea cycle by Leuthardt (18–20) and Bach (21).

SUMMARY

1. The conversion of citrulline to arginine by transimination with glutamic acid has been demonstrated to occur in rat liver homogenates fortified with adenosine triphosphate, cytochrome c, and magnesium ions.

2. Glutamic acid appears to be an obligatory intermediate in the introduction of ammonia at the citrulline $\rightarrow$ arginine step of the urea cycle.

3. The synthesis of urea from glutamic acid, glutamic acid plus ammonia, and ammonia by liver slices and homogenates has been studied and the findings discussed.

BIBLIOGRAPHY

1. Krebs, H. A., and Henseleit, K., Z. physiol. Chem., **210**, 33 (1932).
2. Krebs, H. A., Z. physiol. Chem., **217**, 191 (1933).
3. Krebs, H. A, Biochem. J., **36**, 758 (1942).
4. Gornall, A. G., and Hunter, A., J. Biol. Chem., **147**, 593 (1943).
5. Evans, E. A., Jr., and Slotin, L., J. Biol. Chem., **136**, 805 (1940).
6. Rittenberg, D., and Waelsch, H., J. Biol. Chem., **136**, 799 (1940).
7. Shemin, D., and Rittenberg, D., J. Biol. Chem., **153**, 401 (1944).
8. Rittenberg, D., Schoenheimer, R., and Keston, A. S., J. Biol. Chem., **128**, 603 (1939).
9. Borsook, H., and Dubnoff, J. W., J. Biol. Chem., **141**, 717 (1941).
10. Cohen, P. P., and Hayano, M., J. Biol. Chem., **166**, 239 (1946).
11. Cohen, P. P., and Hayano, M., Federation Proc., **5**, 129 (1946).

12. Archibald, R. M., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 155 (1943).
13. Archibald, R. M., *J. Biol. Chem.*, **156**, 121 (1944).
14. Krebs, H. A., *Biochem. J.*, **32**, 108 (1938).
15. von Euler, H., Adler, E., Günther, G., and Das, N. B., *Z. physiol. Chem.*, **254**, 5 (1938).
16. Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154**, 129 (1944).
17. Krebs, H. A., Annual review of biochemistry, Stanford University, 5, 247 (1936).
18. Leuthardt, F., *Z. physiol. Chem.*, **252**, 238 (1938).
19. Leuthardt, F., *Biochem. Z.*, **299**, 280 (1938).
20. Leuthardt, F., and Glasson, R., *Helv. chim. acta*, **25**, 630 (1942).
21. Bach, S. J., *Biochem. J.*, **33**, 1833 (1939).

PEPTIDE BOND SYNTHESIS

I. THE FORMATION OF p-AMINOHIPPURIC ACID BY RAT LIVER SLICES*

By PHILIP P. COHEN and R. W. McGILVERY

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

The dynamic state of the amino acids in tissues makes the direct study of peptide bond synthesis difficult. For this reason, we sought a model reaction that would be free from this objection. Borsook and Dubnoff (1) have suggested that the formation of hippuric acid closely resembles, from both chemical and thermodynamic standpoints, the synthesis of peptides. Even with their improved methods, analytical difficulties would make a study of this reaction tedious. We have chosen to study the formation of the analogue, p-aminohippuric acid since we were able to develop a reasonably accurate analytical method for its determination.

The production of p-aminohippuric acid (PAH) has further interest because p-aminobenzoic acid (PAB) has not only been reported as a constituent of natural peptides (2, 3) but is generally distributed in a difficultly hydrolyzable form in native proteins (4, 5) suggestive of peptide linkages. PAB and PAH have the further advantage of low toxicity (6, 7).

This study was designed to explore the kinetics and optimum conditions of the formation of PAH from PAB and glycine by rat liver slices.

Analytical

The method developed for the determination of PAH depends upon the differential extraction of PAB from PAH by ether, and subsequent colorimetric analysis by a modification of the method of Bratton and Marshall, which has been shown to be valid for PAH and PAB (8, 9). Smith *et al.* had previously reported in abstract (10) a separation with ethylene dichloride, but details were not available to us at the time this study was initiated.

Briefly, the protein in the sample is precipitated with trichloroacetic acid, the sample is neutralized and diluted to a convenient range, buffered to pH 3.95, extracted twice with ether, extracted with benzene to remove the ether, and then analyzed.

Preparation of Sample—1 ml. of a solution containing from 50 to 1500 γ per ml. of total PAB-PAH (all PAH weights in this paper will be in terms of the equivalent amount of PAB) is added to 4.0 ml. of 0.200 N trichloroacetic acid. The precipitated proteins are centrifuged off, and 2.0 ml. of

the supernatant are added to 3.1 ml. of 0.100 N NaOH.   Sufficient distilled water is added to bring the total PAB-PAH concentration down to less than 15 γ per ml. (8 to 10 γ per ml. are desirable).

An appropriate aliquot of the order of 1 ml. is accurately measured into a colorimeter tube for total analysis.

Extraction of PAB—1 ml. of a buffer, pH 3.95 (made by adding 0.1 M citric acid to 0.2 M disodium phosphate to the correct pH), is added to 2.0

TABLE I

Extraction of p-Aminobenzoic Acid (PAB) and p-Aminohippuric Acid (PAH) under Various Conditions

Total volume of all samples brought to 3.0 ml. after the addition of 1 ml. of pH 3.95 citrate-phosphate buffer.

Conditions of extraction	PAB added	PAH added	Total not extracted	
	γ	γ	γ	*per cent*
(a) Extracted twice for 10 min.	1026		17.0	1.6
with 15 ml. ether	684		11.0	1.6
	171		3.0	1.7
	85		1.4	1.6
		68	57.0	84
		34	31.4	92
		3.4	3.3	97
(b) Extracted twice for 5 min.		68	54.6	80
with 15 ml. ether		68	55.2	81
		68	54.6	80
(c) Same as (b) with additional		68	58.2	86
2 min. extraction with 15 ml.		68	58.2	86
benzene		68	58.8	87
(d) Extracted twice for 5 min.	68		1.2	1.8
with 10 ml. ether; once for 2	68		1.3	1.9
min. with 15 ml. benzene	68		1.2	1.8
		68	61.2	90
		68	61.8	91
		68	61.8	91

ml. of the diluted and neutralized sample in a 60 ml. bottle fitted with a cork and a short length of glass tubing.   10 ml. of ether, freshly distilled from stannous chloride, are added and the bottle is stoppered and shaken for 5 minutes on a mechanical shaker at 200 oscillations per minute with a 4.5 cm. throw at the top of the bottles.   The ether is aspirated off with the aid of a capillary tip until the underlying aqueous phase begins to come over (about 1 ml. of ether remaining).   10 additional ml. of ether are added, and the shaking and aspiration repeated.   15 ml. of benzene are added, and

shaking is continued for 2 minutes.   2 ml. of the aqueous phase are pipetted into a colorimeter tube for PAH analysis.

A more efficient extraction removes a relatively high and variable amount of PAH (Table I, (a)).   The additional extraction by benzene raises the analyses for PAH by the amount to be predicted from the solubility of ether in water (Table I, (b) and (c)).   These and experiments with less efficient conditions led to the adoption of the method used (Table I, (d)), in which an optimum balance is attained between the extraction of the PAB and PAH.

Table II shows the recovery of PAH over a wide range of PAB concentrations in which the percentage of PAH is from 5 to 75 per cent of the total PAB-PAH.   These and many subsequent determinations in the course of our studies demonstrate that the method has at least 5 per cent accuracy over the range of concentrations we use.   When the amount of PAH ex-

TABLE II

Test of Accuracy over Range of Relative Concentrations of p-Aminobenzoic Acid (PAB) and p-Aminohippuric Acid (PAH)

All samples brought to a total volume of 3.0 ml. after the addition of 1 ml. of pH 3.95 citrate-phosphate buffer, extracted twice with 10 ml. of ether for 5 minutes and once with 15 ml. of benzene for 2 minutes.   PAH found calculated on basis of 2 per cent PAB and 91 per cent PAH not extracted.   All weights are in terms of PAB.

added	PAH added	Total unextracted	PAH found	
γ	γ	γ	γ	*per cent*
65.0	3.4	4.5	3.5	103
51.0	17.0	16.8	17.4	102
34.0	34.0	31.8	34.3	101
17.0	51.0	46.8	51.1	100

ceeds 30 per cent of the total PAB-PAH present, the error is not over 2 per cent.

Colorimetric Analysis—The total volume of the solutions in the colorimeter tubes is brought to 10 ml. with 0.1 N HCl.   1 ml. of each of the following solutions is added at 3 minute intervals with shaking: (1) 0.1 per cent c.p. sodium nitrite, (2) 0.5 per cent ammonium sulfamate, (3) 0.1 per cent N-(1-naphthyl)ethylenediamine dihydrochloride.   After 10 minutes the solutions are compared against a reagent blank in a spectrophotometer at 540 mμ, and the amounts (as PAB) determined from a calibration curve.

Calculations—Since the analysis after the extraction represents 2 per cent of the PAB and 91 per cent of the PAH (Table I, (d)) in the sample, and the total PAB-PAH is known by analysis, the true amount of PAH may be obtained by this equation.

$$PAH = \frac{\text{(total after extraction)} - 0.02\text{(total before extraction)}}{0.89}$$

We have found it convenient to construct a three-dimensional chart, with the total before extraction *in the aliquot analyzed for PAH* as horizontal coordinates, the true PAH as vertical coordinates, and the total after extraction as a set of coordinates at a slight angle from the vertical. Both the total analysis and the PAH must be multiplied by the appropriate dilution factors to obtain the concentration in the original sample.

A set of fifteen determinations can be completed within 2 hours.

Procedure

All experiments except the large scale isolation studies were run in Warburg flasks at 38°. The gas phase was pure oxygen unless otherwise stated. KOH solution was added to the center wells. All media were made by adding isotonic neutral solutions of the substrates to phosphate-buffered Krebs' Ringer's solution, and 4 ml. of medium were used per flask. Rat tissues were used for all experiments. The flasks were chilled on ice before addition of the tissue slices. After completion of an experiment, the tissue slices were rinsed in water, blotted, and placed in tared cups for drying and weighing.

The PAB used was a commercial product, taken up in hot alcohol, decolorized with norit, and recrystallized from an alcohol-water mixture, forming long white needles. The PAH was generously supplied by Dr. Karl Beyer of Sharp and Dohme, Inc., and was a white powder melting sharply at 198.5°, chromogenically equivalent to the PAB. Commercial c.p. glycine was used.

Results

Concentration of PAB—The amount of PAH per mg. of dry liver produced in 4 hours in the presence of 0.01 M glycine rose linearly with increasing PAB concentration up to about 0.001 M and then leveled rapidly (Fig. 1). In other experiments not shown we could demonstrate no significantly greater production beyond 0.0025 M.

Concentration of Glycine—Increasing the glycine concentration beyond 0.003 M did not greatly increase the formation of PAH from 0.001 M solution of PAB in 4 hours (Fig. 2). However, we used 0.01 M glycine solutions in our experiments to insure that it was not the limiting factor for the reaction. It is interesting to note that in the absence of added glycine 2.6 γ of PAH were produced per mg. of dried liver, indicating that at least 1.4 γ of endogenous glycine were available per mg. of dry weight. This amount of glycine

is of the same order as that found to be available for the production of glycocyamine from added arginine (11).

Amount of Tissue—Up to a dry weight of tissue of 20 mg. in 4 ml. of medium, 0.00094 M to PAB and 0.01 M to glycine, the amount of PAH pro-
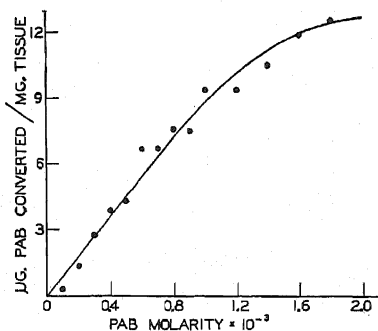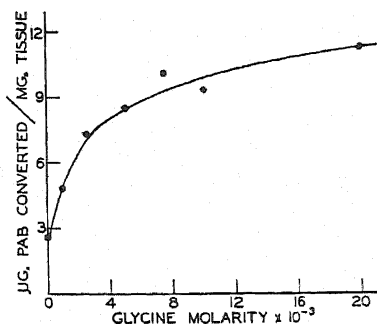
FIG. 1   FIG. 2

FIG. 1. *p*-Aminohippuric acid produced per mg. of dry weight of rat liver with increasing *p*-aminobenzoic acid concentrations. 22 to 42 mg. of dry weight of tissue in 4 ml. of medium, 0.01 M to glycine. Incubated 4 hours at 38° under 100 per cent O_2.

FIG. 2. *p*-Aminohippuric acid produced per mg. of dry weight of rat liver with increasing glycine concentrations. 22 to 39 mg. of dry weight of tissue in 4 ml. of medium, 0.001 M to *p*-aminobenzoic acid. Incubated 4 hours at 38° under 100 per cent O_2.
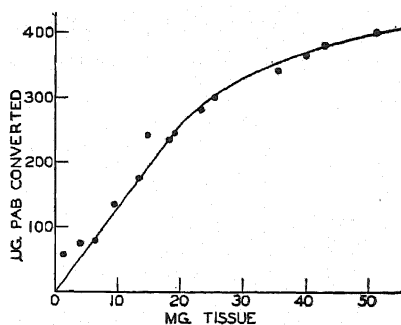
FIG. 3. Production of *p*-aminohippuric acid by increasing amounts of rat liver slices. 4 ml. of medium, 0.01 M to glycine, containing 516 γ of *p*-aminobenzoic acid (0.00094 M), per flask. Incubated 4 hours at 38° under 100 per cent O_2.

duced per mg. of tissue in 4 hours was constant (Fig. 3). With greater weights, the efficiency markedly fell off until 40 mg. of dry weight produced only 9.25 γ of PAH per mg. of tissue, while 20 mg. of dry weight produced 12.7 γ per mg. of tissue.

Isolation of End-Product—Because of its amphoteric character, the isolation of small amounts of PAH is difficult. We found that norit will quantitatively adsorb it from a protein-free solution at pH 4.0, but we were not able to find a satisfactory method of quantitatively eluting it from the adsorbent. For this reason we chose to isolate the product as the *p*-(*p*-hydroxyphenyl)azo derivative. Five 250 ml. Erlenmeyer flasks were fitted with stoppers and glass tubing for continuous passage of gas. In each flask were placed 100 ml. of medium, 0.0015 M to PAB and 0.01 M to glycine. Rat livers were sliced as before, and approximately 300 to 500 mg. (dry weight) of tissue were placed in each flask. The flasks were then incubated at 38° in a constant temperature bath with shaking until no more PAH was formed (7.5 to 8 hours). During the incubation, a slow stream of oxygen was maintained through them. In a typical run, a total of 22 mg. of PAB was converted to PAH, the efficiency of conversion being less than that of the smaller scale experiments.

After removal of the tissue, trichloroacetic acid, dissolved in a minimum of water, was added to bring the concentration up to 3 per cent. The precipitated proteins were centrifuged off, and the resultant clear yellow solution neutralized to pH 4.0 with solid sodium citrate. The solution was then extracted with ether in a separatory funnel until less than 0.5 mg. of free PAB remained.

The solution was chilled to 1°, 25 ml. of 12 N HCl were added, and a 1 per cent solution of sodium nitrite was added in small portions until a test for nitrous acid with iodide-starch paper remained positive. Solid ammonium sulfamate was then added in small portions until no free nitrous acid remained. This solution was slowly poured with vigorous stirring into an ice-cold solution of 200 mg. of phenol in 100 ml. of 5 N NaOH. The orange-colored azo compound formed immediately. The solution was made acid with sulfuric acid and extracted with portions of ether until the ether remained colorless. The ether was extracted with 100 ml. of saturated sodium bicarbonate solution (more may be necessary if much acid has been carried over). This bicarbonate solution was acidified and extracted with 200 ml. of ether, and the ether extracted with 15 ml. of 5 per cent bicarbonate. The final bicarbonate solution was acidified with 10 N sulfuric acid, and the bright orange precipitate centrifuged off, washed with water, recrystallized from an alcohol-water mixture, dried, and weighed. Yield, 24 mg., which represents 50 per cent of the PAH formed in the incubation medium. Darkens at 243°, melts with decomposition at 246°.

For purposes of comparison, *p*-(*p*-hydroxyphenyl)azobenzoic acid and *p*-(*p*-hydroxyphenyl)azohippuric acid were synthesized as follows: 0.7 mM of PAB or PAH was dissolved in 20 ml. of 0.1 N HCl. A 10 per cent excess of sodium nitrite dissolved in a small amount of water was added slowly to

the solution with stirring at 1°. The solution was allowed to stand for 15 minutes, and ammonium sulfamate equivalent to the nitrite was added. The resultant solution was slowly poured into a solution of 10 per cent excess phenol in 30 ml. of 0.1 N NaOH at 1° with vigorous stirring. The solution was acidified with sulfuric acid and then neutralized with solid bicarbonate. It was then extracted with 3 volumes of ether to remove the excess phenol. After again acidifying and standing at 1° overnight, the resultant azo compound was centrifuged off, washed with water, and recrystallized from alcohol-water mixtures. The yields were excellent (over 90 per cent in all cases). PAB derivative darkens at 265°, melts with decomposition at 268°. PAH derivative darkens at 244°, melts with decomposition at 246.5°. A mixture of the azo derivative from the tissue experiment and that from known PAH darkened at 244° and decomposed at 246°.

It was found that solutions of the azo derivatives of PAB and PAH are chromogenically equivalent within 1.5 per cent in 0.1 N NaOH solution at 440 mμ. The derivative from the tissue experiment was then compared with the known derivatives, and a chromogenic equivalent weight of 304 obtained (theoretical for p-(p-hydroxyphenyl)azohippuric acid, 299).

In another tissue experiment in which the 26 mg. of PAH produced were adsorbed on norit, as mentioned previously, we were able to remove 10.5 mg. of the PAH by continuous extraction for 48 hours with alcohol. By evaporating the alcohol *in vacuo* and diazotizing a solution of the resultant gummy mass, a more impure sample of the azo derivative was obtained, which darkened at 241° and decomposed at 243°. Upon mixing with the known PAH azo derivative, the mixture darkened at 242° and decomposed at 244.5°.

We consider these experiments to be an identification of the bulk of the end-product as p-aminohippuric acid.

Time Curve—An evaluation of the production of PAH with increasing time is complicated by the accelerated decomposition of liver slices after 3 to 4 hours of shaking, causing the final dry weight of a given amount of wet tissue to fall, and the apparent production per mg. of dried tissue to continue rising. To get a truer picture of the time relationships, we followed the production in one of the large scale runs described in the previous section, in which the concentration of PAB was 0.0015 M and of glycine was 0.01 M. 1 ml. aliquots were withdrawn from a single flask at intervals of time for analysis, and at the end of the run all of the tissue slices were removed for weighing. There was a total of 468 mg. of dry weight of liver slices in 100 ml. of medium. As can be seen in Fig. 4, the production per unit time is constant to about 4 hours, and then begins to fall off. In other runs made with individual Warburg flasks, substantially the same curve

was obtained with 0.001 M PAB media, except that the production per mg. of tissue was at a slightly lower level and apparently continued rising longer because of the decomposition of tissue mentioned.
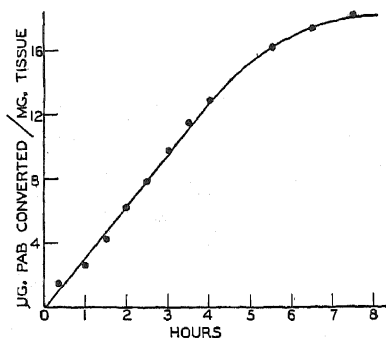

Fig. 4. p-Aminohippuric acid produced per mg. of dry weight of rat liver slices with increasing time.   468 mg. of dry weight of tissue in 100 ml. of medium, 0.0015 M to p-aminobenzoic acid, 0.01 M to glycine.   Incubated at 38° with continuous flushing by 100 per cent O_2.

TABLE III

Effect of Various Concentrations of p-Aminohippuric Acid (PAH) on Conversion of p-Aminobenzoic Acid (PAB)

Each Warburg flask contained 27 to 37 mg. of dry weight of rat liver slices in 4 ml· of medium, 0.01 M to glycine, and was incubated 4 hours at 38° in a 100 per cent O_2 atmosphere.

Initial PAB concentration	Initial PAH concentration	Final PAH concentration	PAB converted to PAH	Total PAB-PAH recovered
$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$	γ per mg. dry weight of tissue	per cent
0.91	0.90	1.44	11.0	98
0.91	0.80	1.41	11.2	100
0.91	0.70	1.33	11.0	100
0.91	0.60	1.17	11.5	100
0.91	0.50	1.10	9.4	95
0.91	0.40	1.03	12.6	98
0.91	0.30	0.96	9.5	100
0.91	0.20	0.84	10.5	100
0.91	0.10	0.74	11.1	99
0.91		0.67	10.8	99
0.91		0.58	10.6	100
	1.00	0.90		93
	1.00	0.92		96

Effect of PAH—An experiment was set up in which increasing amounts of PAH were placed in a medium which was 0.00091 M to PAB and 0.01 M

to glycine (Table III). The reaction was not retarded even when the final concentration of PAH was over three times that of the PAB.

The total recoveries shown are typical of all experiments. That from an experiment with only PAH added was 93 to 96 per cent, and may, in comparison with the recovery of PAB added alone, *i.e.* 99 to 100 per cent, indicate a greater degree of acetylation of PAH than of PAB (10). The magnitude of this possible acetylation reaction is so small that it is without effect on the quantitative significance of our experiments. Since 96 to 97 per cent of the total PAB-PAH recovered is in the form of PAH when PAH

TABLE IV

Formation of p-Aminohippuric Acid (PAH) by Various Rat Tissues

All tissues except testes added as slices to 4 ml. of medium, 0.00102 M to *p*-aminobenzoic acid (PAB), 0.01 M to glycine. Incubated 4 hours at 38° under 100 per cent O_2.

Total recovered		PAB converted	Weight of tissue
	per cent	*γ per mg. dry tissue*	*mg. dry weight*
Liver	92	8.0	36.7
	94	8.7	36.5
Kidney cortex	98	19.3	21.2
	100	22.3	20.3
Testes	90	0.2	75.8
	92	<0.1	66.1
Heart ventricle	100	<0.1	22.7
	101	0.0	22.2
Muscle (thigh)	98	0.0	37.3
	96	0.0	45.1
Brain	100	0.0	17.9
	102	0.0	17.1
Spleen	98	0.0	14.5
	98	0.0	16.3

alone is added, hydrolysis of the PAH formed in these experiments is negligible.

Formation by Other Tissues—Table IV presents the results with several rat tissues with 0.001 M PAB and 0.01 M glycine. All tissues were added as slices, except the testis, in which the testicular capsule was slit open and the tubules teased apart. No tissue other than liver and kidney showed an appreciable formation of PAH. Kidney was roughly 2.5 times as active as liver. A time curve, not shown, was made with kidney and also demonstrated an activity 2.5 to 3 times that of liver. The failure of all tissues to show some synthetic activity is surprising in view of current concepts of the

sites of peptide bond formation. The confinement of primary peptide bond synthesis exclusively to the liver and kidney would be unexpected.

Action of Inhibitors—Various inhibitors were tried (Table V), all being added as the sodium salt in 0.1 M solutions. In the case of cyanide, a solution of cyanide in KOH was used in the center well to prevent distillation of HCN out of the main compartment (12), and in all others KOH alone was placed in the center well. All of the inhibitors were effective in the concentrations used, cyanide, arsenite, iodoacetate, and fluoride being almost completely so.

TABLE V

Action of Inhibitors on Formation of p-Aminohippuric Acid (PAH)

Inhibitors added as sodium salts in 0.1 M solutions. Final volume of medium 4 ml. Concentration of glycine 0.01 M. *p*-Aminobenzoic acid (PAB) concentration 0.00106 M for controls, 0.00105 M for 0.001 M inhibitor solutions, 0.00096 for 0.01 M inhibitor solutions. Incubated 4 hours at 38° under 100 per cent O_2.

Tissue weight		PAB converted	Average inhibition
	mg. dry weight	*γ per mg. dry tissue*	*per cent of average control*
Cyanide 0.001 M	29.6	0.0	
	32.3	0.2	99
Arsenite 0.01 M	21.4	0.7	
	24.0	0.3	95
Iodoacetate 0.01 M	22.2	0.3	
	27.5	0.3	97
Azide 0.001 M	25.9	5.0	
	27.6	5.3	44
Malonate 0.001 M	21.8	6.0	
	26.6	5.8	35
Fluoride 0.01 M	37.4	0.8	
	26.6	1.1	90
Control	19.5	8.8	
	16.0	9.4	

Miscellaneous Observations—In all of the experiments in this section, the PAB concentration was 0.001 M, the glycine 0.01 M, and the run was continued 4 hours. When anaerobic conditions were maintained by gassing with pure nitrogen, the conversion of PAB dropped from a control value in 100 per cent oxygen of 10.1 γ per mg. of dry weight to 0.6 to 0.8 γ per mg. of dry weight of liver. However, lowering the oxygen tension by using air instead of 100 per cent oxygen caused no diminution of production, a value of 11.3 to 11.5 γ per mg. of dry weight being attained as against a control of 11.6 γ per mg. in pure oxygen.

DISCUSSION

The problem of finding a simple model for peptide bond synthesis analogous to *in vivo* protein synthesis has challenged all investigators in this field. The question as to whether the formation of PAH from PAB and glycine represents such a model is not readily answered. The fact that one of the reactants is an α-amino acid and that the end-product has the same type of a linkage as that which occurs in proteins should make this reaction acceptable as a model. However, the problem at this stage is not one of demonstrating true protein synthesis *in vitro*, but rather of detached study as to the kinetics and energetics of a model system.

The Q_{PAH} in our experiments is from 0.5 to 0.6 under the more efficient conditions (the linear portion of the curves in Figs. 1, 3, 4). Harrison and Long (13), in experiments on the regeneration of rat liver protein following fasting, have found that under the most favorable conditions liver tissue *in situ* corresponding in weight to 96 mg. of nitrogen could produce 14 additional mg. of nitrogen in 24 hours. Assuming the liver to be 75 per cent protein on a dry weight basis and the average molecular weight of the amino acids to be 100, the Q for peptide bond formation would be 0.63, a value of the same order as Q_{PAH}.

It is of interest that the reaction cannot proceed anaerobically, nor in the presence of oxidative inhibitors. Evidently, the free energy of the endergonic formation of the peptide bond must be supplied through an oxidative process. Glycine, on the other hand, is regarded as being totally inert in the liver as far as oxidation is concerned (14). Therefore, any scheme that involves the oxidative deamination of glycine is unlikely. The necessity of support by an oxidative metabolism precludes any formation through the reversal of an enzymatic hydrolytic process. It seems highly probable that the formation proceeds through a high energy-phosphorylated intermediate, the phosphorylation being supported by endogenous metabolism. While there is nothing in this study, particularly in the action of inhibitors, to rule out this mechanism, there is also no direct support for it. We hope to investigate the mechanism more directly in a subsequent study.

In contrast to our findings with rat kidney, Borsook and Dubnoff (1) have reported that kidney has only one-third the activity of liver in the synthesis of hippuric acid from benzoic acid and glycine. Since the Q_{PAH} values in our experiments with liver are of the same order as the $Q_{hippuric\ acid}$ values in most of their liver studies, it is not clear as to whether the difference in our findings with kidney is due to a qualitative difference with PAB as compared to benzoic acid, or to differences in experimental conditions in the two studies.

The high activity of kidney cortex is of interest in view of the fact that

PAH has been shown to be effective in lowering the rate of penicillin excretion by the kidney (15).

SUMMARY

1. A method is described for determining p-aminohippuric acid (PAH) in the presence of p-aminobenzoic acid (PAB) by extracting the latter from an aqueous solution with ether.

2. The formation of PAH from PAB and glycine by rat liver slices is demonstrated.

3. The system is at an optimum at 0.001 M PAB, 20 mg. of dry weight of tissue in 4 ml. of medium, and at 4 hours' time.

4. The formation of PAH does not occur anaerobically nor in the presence of oxidative inhibitors.

5. The isolation of the p-(p-hydroxyphenyl)azo derivative of the end-product is described, and a method is given for the synthesis of similar derivatives of PAB and PAH.   The derivative of the end-product is found to be identical with that of PAH.

6. Of the tissues tested, only the liver and kidney were active.   Testes, heart, thigh muscle, spleen, and brain were inactive.   The kidney was 2.5 to 3 times as active as the liver.

BIBLIOGRAPHY

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132,** 307 (1940).
2. Ratner, S., Blanchard, M., Coburn, A. F., and Green, D. E., *J. Biol. Chem.*, **155,** 689 (1944).
3. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, **103,** 667 (1946).
4. Lewis, J. C., *J. Biol. Chem.*, **146,** 441 (1942).
5. Pennington, D., *Science*, **103,** 397 (1946).
6. Scott, C. C., and Robbins, E. B., *Proc. Soc. Exp. Biol. and Med.*, **49,** 184 (1942).
7. Mattis, P. A., Beyer, K. H., McKinney, S. E., and Patch, E. A., *J. Pharmacol. and Exp. Therap.*, **84,** 147 (1945).
8. Eckert, H. W., *J. Biol. Chem.*, **148,** 197 (1943).
9. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, **24,** 388 (1945).
10. Smith, P. K., Bayliss, J. R., Orgorzalek, S., and McClure, M. M., *Federation Proc.*, **5,** 154 (1946).
11. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138,** 389 (1941).
12. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 45 (1945).
13. Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, **161,** 545 (1945).
14. Bach, S. J., *Biochem. J.*, **33,** 90 (1939).
15. Beyer, K. H., Verwey, W. F., Woodward, R., Peters, L., and Mattis, P. A., *Am. J. Med. Sc.*, **209,** 608 (1945).

GLUTAMIC ACID ANTIMETABOLITES: THE SULFOXIDE DERIVED FROM METHIONINE*

By HEINRICH WAELSCH, PHYLLIS OWADES, HERBERT K. MILLER, AND ERNEST BOREK

(From the Departments of Biochemistry, New York State Psychiatric Institute and Hospital and the College of Physicians and Surgeons, Columbia University, New York)

Recently it has been found that the administration of $l(+)$-glutamic acid decreases the number of seizures in some patients suffering from petit mal epilepsy (1, 2) and improves significantly the mental performance of mental defectives of a certain type (3). Since these findings indicate the possibility of an abnormal metabolism of glutamic acid in such subjects, it appears desirable to study the effects of a glutamic acid deficiency in mammals. Such a nutritional state is difficult to achieve because glutamic acid is synthesized in mammals. Therefore, a search for compounds which may interfere with the normal utilization of the amino acid was undertaken. Since a study of antimetabolites is greatly facilitated by the use of an organism for which the metabolite is essential, *Lactobacillus arabinosus* was selected as the test organism.

The effect of homologues, sulfur analogues, and phenyl-substituted derivatives of glutamic acid on the growth of these organisms was studied. α-Aminoadipic acid, aminomalonic acid, and i-β-phenylglutamic acid did not inhibit the growth of the bacteria, but i-γ-methylsufinyl-α-aminobutyric acid (the sulfoxide derived from methionine) and i-γ-benzylsulfinyl-α-aminobutyric acid (the sulfoxide derived from benzylhomocysteine) were effective antimetabolites of glutamic acid. The detailed experimental data obtained in these studies, which have been briefly reported elsewhere (4, 5), are presented.

EXPERIMENTAL

dl-α-Aminoadipic Acid—This compound has been prepared by Sørensen (6) from γ-chlorobutyronitrile and phthalimidomalonic ester. Since adipic acid is now commercially available, a cheaper method of preparation from this source was designed. Diethyl-α-bromoadipate was prepared according to the method of Ingold (7) in a 65 per cent yield. A solution of 314 gm. of the bromo ester in 1 liter of methanol was saturated with

ammonia gas, resaturated after 24 hours at room temperature, and allowed to stand 24 hours longer. The ammoniacal solution was taken to dryness *in vacuo* and the oily residue was boiled with refluxing for 2 hours with 1200 ml. of 6 N HCl. The solution was evaporated to dryness *in vacuo* and the excess of hydrochloric acid was removed by repeated addition and evaporation of water *in vacuo*. The crystalline mass was dissolved in 300 ml. of water, extracted three times with 100 ml. of ether, and the aqueous solution was treated with charcoal. The filtrate was made just alkaline to Congo red with concentrated ammonia (about 60 ml.) and cooled overnight. 125 gm. of α-aminoadipic acid were obtained (69 per cent yield).

For recrystallization 10 gm. of the crystals were dissolved in 50 ml. of water and 11 ml. of 6 N HCl. 11 ml. of 6 N ammonia solution were added and the solution was placed in the ice box. 7 gm. of crystals were obtained. If the amino acid is recrystallized from water or dissolved in ammonia first and then precipitated with acid, the yield is much smaller, owing to the formation of the water-soluble piperidonecarboxylic acid. After two recrystallizations a product was obtained with a melting point of 204° in a bath preheated to 195° (205–206° (6)).

The benzoyl-α-aminoadipic acid was prepared in the usual way, m.p. 180° (184° (6)).

Aminomalonic ethyl ester and i-β-phenylglutamic acid[1] were prepared according to the methods of Snyder and Smith (8) and Harington (9) respectively.

i-γ-Methylsulfinyl-α-aminobutyric acid (*i*-MSO) was prepared by a slight modification of the method of Toennies and Kolb (10). HBr was used instead of HCl to dissolve the methionine, and for neutralization ammonia was used instead of amylamine. Since precipitation of the sulfoxide with acetone (10) yields the product in almost theoretical amounts, any appreciable fractionation of the isomers is excluded.

dl-γ-Methylsulfonyl-α-aminobutyric acid was prepared according to the method of Toennies and Kolb (11).

i-γ-Benzylsulfinyl-α-aminobutyric acid (*i*-BzSO) was prepared from S-benzylhomocysteine (12) in the same manner as the MSO. It was recrystallized from boiling water and was obtained in a yield of over 90 per cent (m.p. 230°).

$C_{11}H_{15}NO_3S$ (241.2). Calculated, N 5.8, S 13.3; found, N 5.9, S 13.6

dl-γ-Benzylsulfonyl-α-aminobutyric acid was prepared by the method of Toennies and Kolb (11). It was recrystallized from boiling water. (Yield 80 per cent; m.p. 246°.)

[1] Prepared by Robert B. Case.

$C_{11}H_{15}NO_4S$ (257.2).   Calculated, N 5.4, S 12.4; found, N 5.4, S 12.4

i-γ-Ethylsulfinyl-α-aminobutyric acid was prepared from ethionine (13) in the same manner as MSO (m.p. 219°).

$C_6H_{13}NO_3S$ (179.1).   Calculated, N 7.8, S 17.9; found, N 7.6, S 18.0

dl-γ-Ethylsulfonyl-α-aminobutyric acid was prepared in the same manner as the methyl derivative (m.p. 239°).

$C_6H_{13}NO_4S$ (195.1).   Calculated, N 7.2, S 16.4; found, N 7.0, S 16.7

Bacteriological Assay—The broth, synthetic medium, and bacteriological techniques described by Hac, Snell, and Williams (14) were employed. All the compounds to be tested were sterilized by filtration.   The concentrated, glutamic acid-free amino acid solution (14) was diluted to a volume of 2.5 ml. either with water or a solution containing the neutralized additional compounds.   Glutamic acid was added in the amounts indicated in each experiment.   The turbidimetric measurements of bacterial growth were carried out with a Coleman Junior spectrophotometer (cuvettes 19 × 105 mm.).

For comparative studies it was imperative to control the size of the inoculum from day to day.   This may be achieved either by growing the culture for inoculation on media containing constant, limiting amounts of glutamic acid, or by diluting the cultures to the same optical density with sterile saline before inoculation.   In most of our experiments 0.04 ml. of bacterial suspension of an optical density of 0.41 to 0.46 was used.

Effect of Various Glutamic Acid Analogues on Growth of Lactobacillus arabinosus—In Table I representative data are shown.   All tubes contained a concentration of 0.82×10^{-3} M $l(+)$-glutamic acid, an amount which yields optimal growth in 20 hours under the conditions of our experiments.   The size of the inoculum was the same throughout.   Under these conditions i-MSO inhibited bacterial growth; the antibacterial index was 50 to 75.   Similar results were obtained with *Lactobacillus casei*.   The antibacterial index is derived from a comparison, after 20 hours of incubation, of optimal with completely inhibited bacterial growth, two states which are stationary.   Comparison of optimal and partially inhibited growth, however, involves the evaluation of two different states, one stationary and the other of active growth.   An analysis of the bacterial growth rate at partial inhibition would be complicated by the fact that the organism metabolizes the sulfoxides.

i-BzSO is more effective as an antimetabolite than i-MSO, though a precise comparison is difficult since some of the benzyl derivative crystallizes out during incubation.   The sulfone derived from *dl*-methionine is

at best one-third to one-half as active as i-MSO. The sulfone derived from dl-benzylhomocysteine could not be tested, as its solubility was too low.

TABLE I

Effect of Various Glutamic Acid Analogues on Growth of Lactobacillus arabinosus
$l(+)$-Glutamic acid 0.82×10^{-3} M, 20 hours incubation.

Compound	Concentration	Bacterial growth, optical density*	Inhibition
	M × 10⁻³		per cent
dl-α-Aminoadipic acid	0	0.44	
	60	0.43	0
Aminomalonic acid ethyl ester	38	0.44	0
i-β-Phenylglutamic acid	36	0.48†	0
Sulfoxide from	0	0.43	
dl-methionine	6	0.42	2
	12	0.37	14
	18	0.32	26
	24	0.25	42
	30	0.19	56
	36	0.03	93
	42	0.01	98
	48	0.01	98
Sulfone from	44	0.25	42
dl-methionine	55	0.25	42
	66	0.17	60
	77	0.10	77
Sulfoxide from dl-benzyl-	18	0.33	23
homocysteine‡	20	0.30	30
	21.5	0.28	35
	23	0.20	53
	25	0.02	95
Sulfoxide from	0	0.44	
dl-ethionine	44	0.42	5
	66	0.41	7
Sulfone from	41	0.43	2
dl-ethionine	62	0.38	14

* Corrected for blank values (medium without glutamic acid).
† Solution developed color on incubation.
‡ Read after addition of HCl to dissolve sediment of antimetabolite.

The sulfoxide and sulfone derived from dl-ethionine are ineffective as antimetabolites in concentrations in which MSO gives complete inhibition. It is interesting that, unlike the parent substance (15), the sulfoxide derived from ethionine does not antagonize methionine.

Effect of Size of Inoculum on Antibacterial Index—The inhibition by i-

MSO was halved by tripling the size of the inoculum and completely coun-teracted by a 10-fold increase (Fig. 1).

Specificity of Glutamic Acid As an Antagonist to Sulfoxides—Fig. 2 shows the reversal of the *i*-MSO and *i*-BzSO inhibition by increasing amounts of $l(+)$-glutamic acid. The response to glutamic acid is highly specific.
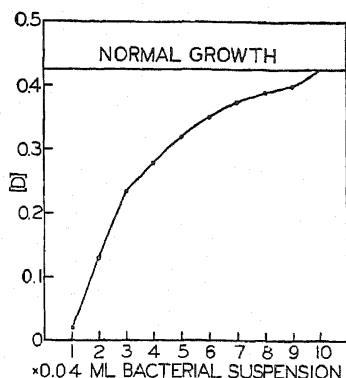
FIG. 1. The reversal of *i*-MSO inhibition of bacterial growth by increased inoculum size. Each tube contained 15 mg. of *i*-MSO $(36 \times 10^{-3}$ M$)$ and 300 γ of glutamic acid $(0.82 \times 10^{-3}$ M$)$. $D = $ optical density.
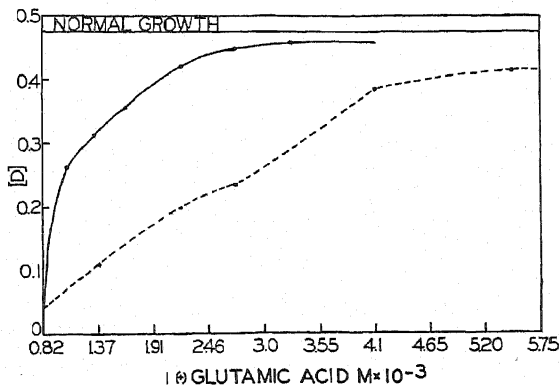
FIG. 2. Reversal of *i*-MSO and *i*-BzSO inhibition of bacterial growth by $l(+)$-glu-tamic acid. $D = $ optical density. Tubes containing 20 mg. of *i*-MSO $(48 \times 10^{-3}$ M$)$ solid line; tubes containing 15 mg. of *i*-BzSO $(23 \times 10^{-3}$ M$)$ broken line.

None of the other amino acids present in the synthetic medium reversed the inhibition when tested separately (tryptophane up to 15 mg. per 2.5 ml., tyrosine up to 10 mg., cystine up to 3 mg., and the others up to 20 mg.). A slight inhibition in the absence of MSO was observed with arginine above 10 mg. per 2.5 ml. Asparagine was found to be ineffective in overcoming the MSO inhibition. Cysteine, which reduces MSO to

TABLE II

Reversal of Inhibition of Bacterial Growth by i-MSO in Presence of Cysteine

Glutamic acid 0.82×10^{-3} M.

i-MSO concentration	Cysteine concentration	Bacterial growth, optical density	Inhibition
$M \times 10^{-3}$	$M \times 10^{-3}$		per cent
0	0	0.45	
0	25	0.46	
0	50	0.45	
48	0	0.01	98
48	2.5	0.01	98
48	5	0.15	67
48	7.5	0.33	26
48	12.5	0.40	11
48	25	0.39	13
48	50	0.40	11

TABLE III

Reversal of Inhibition of Bacterial Growth by i-MSO and i-BzSO in Presence of Glutamic Acid Derivatives

Sulfoxide	Concentration	Glutamic acid concentration	Glutamic acid derivative	Concentration	Bacterial growth, optical density	Inhibition
	$M \times 10^{-3}$	$M \times 10^{-3}$		$M \times 10^{-3}$		per cent
i-MSO	0	0.22			0.19	
"	20	0.22			0.01	96
"	0	0	Glutamine*	0.22	0.19	
"	20	0	"	0.22	0.18	4
"	0	0.82			0.45	
"	48	0.82			0.02	96
"	0	0	Glutamine	0.82	0.45	
"	48	0	"	0.82	0.45	0
"	66	0	"	0.82	0.44	2
"	96	0	"	0.82	0.40	10
"	0	0	Ketoglutaric acid	0.82	0.31	
"	0	0.82	" "	8.2	0.46	
"	36	0	" "	0.82	0.00	100
"	36	0	" "	8.2	0.01	98
"	0	0.82			0.44	
"	36	3.28			0.46	0
"	0	0	Glutathione	0.82	0.42	
"	36	0	"	0.82	0.04	90
"	36	0	"	3.28	0.36	14
i-BzSO	25	0.82			0.07	87
"	25	0	Glutamine	0.82	0.39	13

* We are indebted to Dr. H. B. Vickery and Dr. M. M. Harris for samples of $l(+)$-glutamine.

methionine (10), counteracts the inhibition of bacterial growth by i-MSO (Table II). Although 2 moles of cysteine reduce 1 mole of the sulfoxide, much smaller ratios overcome the growth inhibition.

The growth-inhibiting effect of high concentrations of the methyl-sulfonyl analogue is not overcome by glutamic acid.

Reversal of i-MSO and i-BzSO Inhibition by Glutamic Acid Derivatives (Table III)—If glutamic acid is replaced by an equivalent amount of glutamine, even twice the concentration of i-MSO which inhibits bacterial growth completely in the presence of glutamic acid has practically no influence on the growth of the bacteria. Likewise, concentrations of i-BzSO which inhibit completely in the presence of glutamic acid are ineffective in the presence of glutamine. On the other hand, if glutamic acid is replaced by ketoglutaric acid, i-MSO causes complete inhibition which is not reversed by large amounts of the keto acid.

In accordance with previous findings (14), the bacterial growth with equivalent amounts of glutathione was almost as good as with glutamic acid at optimal concentrations (0.82×10^{-3} M). At such levels of glutamic acid or glutathione the same amount of i-MSO inhibits bacterial growth completely. Glutathione in increased amounts is almost as effective as glutamic acid in reversing the MSO inhibition.

DISCUSSION

Replacing the γ-carboxyl of glutamic acid by the methylsulfinyl group produces a specific antimetabolite, MSO, against glutamic acid in *Lactobacillus arabinosus* and *Lactobacillus casei*. The corresponding sulfonyl derivative is a less active and unspecific inhibitor of bacterial growth and its action may be comparable to that of the aminosulfonic acid antagonists described by McIlwain (16). The antagonism of MSO as an antimetabolite of glutamic acid is highly specific; of the known amino acids only cysteine is at all effective in reversing the inhibition. The antagonism does not depend on the sulfinyl configuration alone, since it is decisively affected by the sulfur substituent other than the α-aminobutyric acid moiety. The methyl and benzyl derivatives are highly active, whereas the ethyl derivative is not active at all. No explanation for this remarkable specificity is offered.

The effect of cysteine is probably related to its ability to reduce MSO to methionine (10). However, much less than the theoretical amount is sufficient to counteract the inhibition. The ease of reduction of MSO by cysteine suggests a similar transformation of the antimetabolite by the normal metabolism of the bacteria, thus offering an explanation for the prevention of the inhibition by large inocula.

Since these sulfoxides have two centers of asymmetry, they exist as four stereoisomers. All the work reported in this communication was carried

out with the inactive material. A study of some of the stereoisomers as antimetabolities will be reported shortly.

The ineffectiveness of i-MSO and i-BzSO as antagonists to glutamine suggests that the sulfoxides prevent the amidation of glutamic acid. Whether the amidation of glutamic acid is the only process with which the sulfoxides interfere is uncertain. Unlike glutamic acid large concentrations of ketoglutaric acid, which supports growth in lieu of glutamic acid in these organisms, are unable to overcome the sulfoxide inhibition. Glutathione, in which the γ-carboxyl is substituted as in glutamine, behaves towards MSO like glutamic acid and not at all like glutamine. It is of interest that MSO, which is able to replace the parent substance (methionine) in the diet of rats (17), acts as a powerful antagonist of another amino acid in bacterial metabolism. The biological significance of this finding cannot be evaluated at this stage, since the occurrence of the sulfoxide derived from methionine in living organisms has not been established.

SUMMARY

The effect of homologues, sulfur analogues, and phenyl-substituted derivatives of glutamic acid was tested on the growth of *Lactobacillus arabinosus*. *dl*-α-Aminoadipic acid, aminomalonic acid, and i-β-phenylglutamic acid do not inhibit the growth of the bacteria. The sulfoxides derived from *dl*-methionine and from *dl*-benzylhomocysteine are effective antimetabolities of glutamic acid, the latter being more active. The sulfone derived from *dl*-methionine is about one-third as effective as the corresponding sulfoxide. The sulfone and sulfoxide derived from *dl*-ethionine are ineffective. $l(+)$-Glutamic acid overcomes specifically the growth inhibition by the sulfoxides. Cysteine counteracts the toxicity of the sulfoxides, probably by reduction. With increasing size of inoculum the degree of sulfoxide inhibition decreases, an observation which suggests that the antimetabolite is made ineffective by the metabolism of the bacteria.

If glutamic acid is replaced by glutamine, even twice the concentration of sulfoxide which inhibits bacterial growth completely in the presence of glutamic acid has little influence on the growth of the bacteria. Ketoglutaric acid is ineffective in overcoming the sulfoxide inhibition, but glutathione is almost as effective as glutamic acid.

BIBLIOGRAPHY

1. Price, J. C., Waelsch, H., and Putnam, T. J., *J. Am. Med. Assn.*, **122**, 1153 (1943).
2. Waelsch, H., and Price, J. C., *Arch. Neurol. and Psychiat.*, **51**, 393 (1944).
3. Albert, K., Hoch, P., and Waelsch, H., *J. Nerv. and Ment. Dis.*, **104**, 263 (1946).
4. Borek, E., Sheiness, P., and Waelsch, H., *Federation Proc.*, **5**, 123 (1945).

5. Borek, E., Miller, H. K., Sheiness, P., and Waelsch, H., *J. Biol. Chem.*, **163**, 347 (1946).
6. Sørensen, S. P. L., *Compt.-rend. trav. lab. Carlsberg*, **6**, 20 (1902).
7. Ingold, C. K., *J. Chem. Soc.*, **119**, 951 (1921).
8. Snyder, H. R., and Smith, C. W., *J. Am. Chem. Soc.*, **66**, 350 (1944).
9. Harington, C., *J. Biol. Chem.*, **64**, 29 (1925).
10. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, **128**, 399 (1939).
11. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, **140**, 131 (1941).
12. Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, **111**, 393 (1935).
13 Dyer, H, M., *J. Biol. Chem.*, **124**, 519 (1938).
14. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
15. Harris, J. S., and Kohn, H. I., *J. Pharmacol. and Exp. Therap.*, **73**, 383 (1941).
16. McIlwain, H., *Brit. J. Exp. Path.*, **22**, 148 (1941).
17. Bennett, M. A., *Biochem. J.*, **33**, 1794 (1939).

THE ISOLATION AND CHARACTERIZATION OF TWO ANTIGENIC FRACTIONS OF PROTEUS OX-19*

BY AARON BENDICH† AND ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

The Weil-Felix reaction (1–3), used in the diagnosis of several of the fevers of the typhus group, presents a very interesting instance of a heterogenetic immunological reaction (4). It is based on the presence in the serum of typhus patients of agglutinins for the O variety of the X-19, X-2, and X-K strains of *Proteus*. A previous communication from this laboratory (5) has dealt with the antigens of *Rickettsia prowazeki*, the etiological agent of louse-borne ("epidemic") typhus. The present study will supply information on the properties of antigenic fractions isolated from *Proteus* OX-19 which is the organism agglutinated by the sera of patients suffering from louse-borne typhus.

The biological significance of the cross-reaction between *Rickettsia* and *Proteus*, organisms extremely different with respect to their taxonomy, is obscure. There does not seem to exist any convincing evidence of a genetic relationship between these organisms.

The chemical composition of the *Proteus* OX-19 antigen responsible for the cross-reaction with typhus convalescent sera has remained largely unexplored. Indications of the presence of specific polysaccharides in *Proteus* extracts have been obtained by a number of workers (6–11). White (12) described an antigenic preparation from a tryptic digest of *Proteus* OX-19 containing two factors: one, labile to alkali, which gave rise, in the rabbit, to an antiserum containing homologous agglutinins for the bacteria; the other, stable to alkali, assumed to be responsible for the Weil-Felix reaction. Castañeda (13–15) reported the separation of two antigenic constituents: the "P factor," unstable in alkali and reacting only with antisera to *Proteus*; and the "X factor," regarded as common to both *Proteus* OX-19 and *Rickettsia prowazeki*, whose reactivity with *Proteus* and typhus sera was reported to be unaffected by boiling in acid or weakly

---

alkaline solution. Mesrobeanu et al. (16–18) described the isolation, by extraction with trichloroacetic acid, of a complex antigen, claimed to be responsible for the Weil-Felix reaction. A discussion of some of the points at which the results presented here are at variance with previous reports will be found later in the paper.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Cultivation of Organisms*</div>

Proteus OX-19 was obtained from the American Type Culture Collection (No. 6380). The best yield of organisms rich in antigenic material was obtained on nutrient agar composed of 2 per cent of Bacto-tryptone, 0.5 per cent of a yeast concentrate,[1] 0.5 per cent of sodium chloride, and 3 per cent of Bacto-agar in tap water.

Roux bottles containing 150 cc. of this medium were sterilized by autoclaving (30 minutes at 15 pounds). An inoculum of 3 cc. of a 24 hour culture was added to each bottle and the cultures were incubated for 24 to 36 hours at 37°. Following the customary bacteriological controls with respect to homogeneity, non-motility, etc., the heavy cultures were removed with physiological saline containing 0.2 per cent of formaldehyde. The organisms were washed three times in the centrifuge with formolized saline at 4000 R.P.M., again suspended in saline, and the suspensions were passed through a very coarse sintered glass filter. This proved an important step, since a considerable quantity of agar particles could thus be removed. The filtrates were dialyzed in the cold against saline containing 0.2 per cent of formaldehyde for 16 hours, against running tap water for 24 hours, and against several changes of ice-cold distilled water for the same period. The suspensions, dried in the frozen state *in vacuo*, yielded the organisms as a fluffy powder (1.5 to 2 gm. per liter of medium). These preparations retained their serological activity even when stored for 2 years at room temperature.

Numerous attempts to use liquid media gave results that were far from encouraging. When agar was omitted from the culture medium described above, the yield of organisms, even with efficient aeration, was about 0.1 gm. per liter. The inclusion of glucose diminished even this yield.

A culture fluid of the following composition, developed in collaboration with Dr. S. Francis, gave good growth (about 0.75 gm. of dry bacteria per liter), but was not employed in the present study, since only one-tenth of the normal yield of antigenic material could be obtained from the organisms: ammonium citrate 5.0 gm., Na_2CO_3 3.0 gm., NaCl 2.0 gm., $MgSO_4 \cdot 7H_2O$

<hr>

[1] Brewers' yeast extract, type 3, obtained through the courtesy of Standard Brands Incorporated.

0.4 gm., Casamino acids (Difco, technical) 2.0 gm., KH_2PO_4 3.0 gm., ferric ammonium citrate 0.05 gm., $l(+)$-cysteine hydrochloride 0.11 gm., thiamine hydrochloride 5.0 mg., nicotinic acid 0.02 mg., tap water to 1 liter.

Antisera to Proteus OX-19

Several rabbits received each a total of 7.56 mg. of dried organisms (0.6 mg. N) intravenously in graded doses over a period of 3 weeks. The antisera were obtained 1 week after the last injection. Suspensions of *Proteus* OX-19 in saline (corresponding in turbidity to No. 3 of McFarland's nephelometric scale (19)) were agglutinated with equal volumes of the antisera at a dilution end-point of 1:5120. The antisera (containing 0.01 per cent of ethyl mercurithiosalicylate) were stable for more than 1 year in the refrigerator. Human convalescent epidemic and murine typhus sera[2] diluted to 1:640 to 1:1256 agglutinated suspensions of these organisms. A suspension of 0.24 mg. per ml. of *Rickettsia prowazeki*, killed with formalin, extracted with ether,[3] and heated for 30 minutes at 75° (13), was agglutinated by both the *Proteus* antisera and the epidemic typhus sera (diluted 1:1280).

Isolation of Antigens by Digestion with Trypsin

After preliminary experiments with several enzymes (crude and crystalline trypsin, crystalline chymotropsin, moccasin venom, malt diastase), crystalline trypsin was chosen. A preliminary ether extraction of the bacteria did not increase the efficiency of the tryptic digestion.

Tryptic Digestion—In a typical experiment, 5.0 gm. of dry organisms, suspended in 400 cc. of 0.1 M borate buffer (pH 7.8) containing ethyl mercurithiosalicylate 1:10,000, were treated with 3.2 mg. of crystalline trypsin for 60 hours at 37°. The mixture was centrifuged and the residue resuspended in 200 cc. of buffer and treated with an additional 1.6 mg. of trypsin for 24 hours. After thorough dialysis, the combined supernatants and washings were concentrated by pervaporation and the extracted material (Fraction T) was recovered in a yield of 26 to 28 per cent of the bacteria.[4] The residual bacterial débris amounted to 22 to 26.5 per cent of the organisms employed. The origin of the organisms (nutrient agar or nutrient broth) did not influence the yield.

---

[2] Obtained through the courtesy of Dr. I. Bengtson of the National Institute of Health and of Colonel H. Plotz of the Army Medical School.

[3] We are grateful to Dr. L. A. Chambers of the University of Pennsylvania for this preparation.

[4] Evaporation of the water *in vacuo* in the frozen state was invariably used for the drying of antigenic fractions or for their recovery from aqueous solutions.

Fraction T contained 12.5 per cent N (Kjeldahl), 0.85 per cent P (20), reducing sugars (as glucose) without hydrolysis 7.2 per cent, and, after hydrolysis for 3 hours with 1 N HCl at 100°, 12.7 per cent (Hagedorn-Jensen). The electrophoretic analysis[5] of a 1.3 per cent solution in borate buffer of ionic strength 0.2 and pH 8.5 revealed two components; viz., a small fraction (22 per cent of the total) with a descending mobility of -8.9×10^{-5} sq. cm. per volt per second which was inactive serologically, and a large component (78 per cent of the total) with a descending mobility of -8.3. This major component in an initial concentration of 0.07 mg. of N per cc. gave precipitation at 1:16 dilution with human endemic typhus serum (diluted 1:10) and at 1:4 dilution with Proteus OX-19 antiserum (1:10).

When Fraction T was heated in saline solution for 1 hour at 100°, its reactivity with typhus serum was destroyed, but its reactivity toward Proteus antiserum was hardly impaired.

High Speed Centrifugal Fractionation of Fraction T—When a 1.0 per cent solution of Fraction T in 0.9 per cent NaCl was subjected to centrifugation at 20,000 R.P.M. $(31,000g)$ for $\frac{1}{2}$ hour, an almost clear supernatant (containing Fraction T-1, 70 per cent of the starting material) and a sediment (Fraction T-2, 22 per cent of the starting material) were obtained.[6] The turbid saline suspension of the sediment was centrifuged at 8000 R.P.M. $(5000g)$ for 10 minutes to remove a small amount of particulate matter and then spun at 20,000 R.P.M. for $\frac{1}{2}$ hour. The fractions were obtained as white fluffy solids after thorough dialysis.

Both fractions gave positive Molisch and Sakaguchi tests, and negative Hopkins-Cole tests. The biuret and xanthoproteic reactions were positive with Fraction T-1, but faint with Fraction T-2. Only Fraction T-1 gave a positive Millon reaction. Some of the properties of these substances are listed in Table I. It will be seen that Fraction T-2 showed reactivity with typhus serum, but lost it on being heated.

Isolation of Antigens by Extraction with Trichloroacetic Acid

Extraction—In a typical experiment, 2.0 gm. of dry organisms were suspended in 280 cc. of 0.1 N trichloroacetic acid and kept at 0° for 16 hours with continuous agitation. The pH of the suspension remained at 1.5. The mixture was centrifuged in the cold, the sediment washed three times, and the combined turbid extracts as well as the bacterial débris were dialyzed for 2 days against running tap water and for 1 day against a few changes of cold distilled water. Fraction C, recovered from the extract,

---

[5] We are highly indebted to Dr. D. H. Moore for the electrophoresis experiments.

[6] All high speed centrifugations were carried out in a refrigerated International centrifuge equipped with a multispeed attachment.

amounted to about 7.4 per cent of the bacteria used; the débris amounted to 90 per cent. Fraction C gave opalescent solutions in water and saline and reacted with typhus serum (1:10) in a dilution of 1:32,000, but lost this property after heat treatment. It also precipitated with anti-*Proteus* serum (1:10) at a dilution of 1:128,000 and, after heating, at a dilution |of 1:64,000.

Fractionation by High Speed Centrifugation—The extract containing Fraction C could be fractionated by high speed centrifugation. The dialyzed extract was concentrated by pervaporation to about 100 cc. Centrifugation at 31,000g for 1 hour caused the separation of a translucent

TABLE I

Antigens Prepared by Digestion of Proteus OX-19 with Trypsin

Fraction	N	P	Reducing sugars (as glucose)		Electrophoretic analysis*		Precipitation end-points†			
			Without hydrolysis	After 3 hrs. hydrolysis with N HCl	Mobilities	Area	*Proteus* OX-19 antiserum		Human endemic typhus serum	
							Intact antigen	Heated antigen	Intact antigen	Heated antigen
	per cent	*per cent*	*per cent*	*per cent*	$u \times 10^5$	*per cent*				
T-1	11.0	1.4	7.5	15.7	−4.9	21	1:123,000	1:7700	Negative	Negative
					−6.6	30				
T-2	5.8	2.1	7.0	15.8	−8.0	49	1:127,000	1:15,000	1:63,000	"
					−6.0	100				

* The substances were examined in 1 per cent solutions in borate buffer of pH 8.5 (ionic strength, 0.2). Mobilities and relative areas are calculated from the descending boundaries. Both fractions were heterodisperse in the analytical ultracentrifuge.

† The sera were diluted 1:10. Stability to heat was tested by keeping the antigens, dissolved in neutral physiological saline, at 100° for 1 hour.

jelly from a slightly opalescent supernatant. The sediment was washed two times in the centrifuge with small portions of distilled water. The combined supernatants yielded Fraction C-1 as a fluffy white felt amounting to about 4.3 per cent of the bacteria. The sediment was freed of some contaminating particulate material by centrifugation at 5000g for 10 minutes; it gave Fraction C-2, about 1.2 per cent of the starting bacteria. This substance dissolved readily in water or saline, the turbid solutions showing marked streaming upon agitation. In the dry state the preparations retained their serological activities for more than 1 year even at room temperature.

The examination of Fraction C-1 in the Tiselius electrophoresis cell in borate buffer at pH 8.5 revealed the presence of two components with

descending mobilities and relative proportions as follows: (1) −6.1 (67 per cent) and (2) −7.2 (33 per cent). Under the same conditions, Fraction C-2 migrated as a single component with a descending mobility of −6.4 × 10⁻⁵ sq. cm. per volt per second. Both fractions were polydisperse in the analytical ultracentrifuge.

Purification—The two components of Fraction C-1 could be separated by fractional precipitation with alcohol. To a 2 per cent aqueous solution of Fraction C-1 kept in an ice bath, cold absolute ethanol was added dropwise with constant stirring, when, at an alcohol concentration of 67 per cent by volume, a white flocculent precipitate formed which was removed by centrifugation after chilling overnight. The sediment, which amounted to 60 to 65 per cent of Fraction C-1 (or 2.6 to 2.8 per cent of the original bacteria), was dissolved in cold water, precipitated with cold alcohol, and dried. This substance is designated *Fraction C-11*. The remaining material, soluble in 67 per cent alcohol, was precipitated from solution upon the addition of alcohol to 80 per cent concentration (by volume), to give *Fraction C-12*. This fraction, completely inactive serologically with both *Proteus* and typhus sera, was not studied further. Both fractions were homogeneous electrophoretically and had the following mobilities (in borate buffer at pH 8.5): Fraction C-11, −6.26 (descending), −6.82 (ascending); Fraction C-12, −6.28 (descending), −6.49 (ascending).

When fractionation of Fraction C-2 was attempted in the same manner, at least 95 per cent was precipitated unchanged serologically at an alcohol concentration of 67 per cent. Hence, for later study, Fraction C-2 was not further purified.

Dried preparations of Fractions C-11 and C-2 appeared to dissolve readily in water or saline, but the solutions deposited a considerable sediment on centrifugation for 10 minutes at 4300 R.P.M., probably owing to aggregation during the drying process. Homogeneous solutions which no longer sediment in the centrifuge at 4300 R.P.M. can be obtained if the antigen suspensions are vigorously shaken for a day or two in the refrigerator.

Chemical Properties—Both fractions, C-2 and C-11, were free of sulfur. No spectroscopic evidence for the presence of a nucleic acid could be obtained. The customary tests for purines and for desoxyribose nucleic acid likewise were negative.

Both substances gave, before hydrolysis, a positive Sakaguchi test for arginine and weakly positive biuret and ninhydrin reactions. Negative tests with the Millon and Hopkins-Cole reagents pointed to the probable absence of tyrosine and tryptophane. Following a hydrolysis with 4 N hydrochloric acid at 100° for 8 hours, both fractions showed a strong ninhydrin reaction, but the Hopkins-Cole, Millon, and biuret reactions and also the Pauly test for histidine were negative.

The Molisch reaction was positive; the keto sugar test of Seliwanoff, the

Tollens-Neuberg reaction for uronic acids, the Rosenthaler test for methyl pentoses, the usual reactions for pentoses, such as the orcinol reaction according to Bial, the phloroglucinol, and the McCance (21) tests, were all negative (22). The reaction for amino sugars according to Elson and Morgan (23) was strongly positive after the acid hydrolysis of the substances.

Both fractions contained lipides; the nature of this material will be discussed later.

The analytical properties of several representative preparations are summarized in Table II. N analyses were, unless noted otherwise, carried out by the Kjeldahl procedure; P was determined colorimetrically (20); the

TABLE II

Per Cent Composition of Antigens Prepared by Extraction of Proteus OX-19 with Trichloroacetic Acid

Fraction	Preparation No.	Ash	C	H	N	Amino N		α-Amino acid N after 16 hrs. hydrolysis with 6 N HCl	P	Reducing sugars (as glucose)		Glucosamine after 16 hrs. hydrolysis with 6 N HCl	Acetyl	Total lipides
						Without hydrolysis	After 16 hrs. hydrolysis with 6 N HCl			Without hydrolysis	After 3 hrs. hydrolysis with N HCl			
C-11	VII	7.3	43.9	7.7	4.3	0.62			2.1	2.8	33.7			
	VIII				4.6	0.65			2.3	3.0	39.0			
	XIII	6.9			5.1		4.1	1.4	2.5	2.7	37.5	23.0		
	XV				5.0				2.4		37.8	23.8	6.0	2.8
C-2	VII	7.1	47.6	7.7	5.1	0.75			2.5	3.9	24.2			
	VIII				4.8	0.68			2.3	3.2	29.3			
	XIII	7.2			5.1		4.4	2.4	2.5	2.6	25.2	11.2		
	XV				5.1				2.4		26.0	10.2	1.9	11.1

reducing sugars were estimated by the Hagedorn-Jensen method. The procedures used for the determination of glucosamine and acetyl groups will be discussed later in the paper.

Immunological Properties of Fractions C-11 and C-2

Preparation of Antisera—Three rabbits were injected intravenously with a total of 0.3 mg. of Fraction C-11 and another set with 0.28 mg. of Fraction C-2 during the course of 3 weeks; the antisera obtained from bleedings by cardiac puncture 1 week after the last injection were preserved in the ice box with 0.01 per cent of ethyl mercurithiosalicylate. A suspension of Proteus OX-19 (No. 3 on the McFarland scale) was agglutinated by the addition of the anti-Fraction C-11 serum at a dilution end-point of 1:5120 and by the addition of anti-Fraction C-2 serum at a dilution of 1:1280.

A suspension of *Rickettsia prowazeki* (0.24 mg. per cc.), treated as described above, was agglutinated by the anti-Fraction C-2 serum diluted 1:1280, but the agglutination by the anti-Fraction C-11 serum, even with 1:40 to 1:80 dilutions, was doubtful. In Table III, the precipitation end-points (antigen dilutions) obtained with both antigenic fractions are given.

TABLE III

Precipitation End-Points of Proteus OX-19 Antigens

Fraction	Final antigen dilutions showing precipitation*			
	Epidemic typhus serum	*Proteus* OX-19 antiserum	Fraction C-11 antiserum	Fraction C-2 antiserum
C-11	No ppt.†	1:64,000	1:256,000	1:32,000
C-2	1:32,000	1:64,000	1:128,000	1:256,000

* The sera were employed in a 1:10 dilution.
† Even in an antigen dilution of 1:1000, no precipitation was observed.

TABLE IV

Antibody Nitrogen Precipitated from 1 Cc. of Anti-Fraction C-11 Rabbit Serum by Varying Amounts of Fractions C-11 and C-2

Fraction	Antigen N added	N in ppt.	Antibody N pptd.	Supernatant tests with			
				Anti-Fraction C-11	Anti-Fraction C-2	Fraction C-11	Fraction C-2
	mg.	*mg.*	*mg.*				
C-11	0.002	0.192	0.190	−	−	+++	+++
	0.006	0.293	0.292	−	−	++	+
	0.010	0.378	0.368	±	±	±	±
	0.014	0.392		+	+	−	−
	0.020	0.402		++	++	−	−
	0.030	0.350		+++	+++	−	−
C-2	0.004	0.220	0.216	−	−	+++	+++
	0.005	0.265	0.260	−	−	++	+
	0.009	0.352	0.343	−	−	±	±
	0.018	0.364	0.346	±	±	−	−
	0.026	0.322		+	+	−	−
	0.035	0.306		+++	+++	−	−

Neither the anti-Fraction C-11 nor the anti-Fraction C-2 serum agglutinated suspensions of *Proteus vulgaris* (grown on nutrient agar).

Quantitative Evaluation of Precipitation Reactions of Fractions C-11 and C-2—The methods used in this study essentially followed those of Heidelberger and Kendall (24). When the precipitation of Fractions C-11 and C-2 by the anti-Fraction C-11 rabbit serum was examined (Table IV),

the supernatants, following the precipitation of Fraction C-2, were found not to contain excess antigen and antibody simultaneously. The supernatants from the precipitation of Fraction C-11, however, exhibited a very narrow zone in which both antigen and antibody were present. On the other hand, when both antigens were examined with anti-Fraction C-2 serum (Table V), the supernatant tests revealed a considerable zone in which antigen and antibody remained in excess at the same time.

Reactions of Proteus OX-19 and Fractions C-11 and C-2 with Antiserum to Proteus OX-19—To 1 cc. portions of anti-*Proteus* OX-19 serum, kept at 37°, were added small successive amounts of *Proteus* OX-19 and Frac-

TABLE V

Antibody Nitrogen Precipitated from 1 Cc. of Anti-Fraction C-2 Rabbit Serum by Varying Amounts of Fractions C-11 and C-2

Fraction	Antigen N added	N in ppt.	Supernatant tests with			
			Anti-Fraction C-11	Anti-Fraction C-2	Fraction C-11	Fraction C-2
	mg.	*mg.*				
C-11	0.003	0.099	−	−	++	+++
	0.006	0.140	±	±	++	+++
	0.011	0.160	+	+	++	++
	0.019	0.171	+++	+++	++	+
	0.027	0.174	+++	+++	+	+
	0.032	0.177	+++	+++	±	±
C-2	0.003	0.097	+	±	+++	+++
	0.006	0.122	++	+	+++	+++
	0.012	0.150	+++	++	+++	+++
	0.024	0.175	+++	++	+++	+++
	0.029	0.182	+++	+++	++	++
	0.035	0.185	++++	++++	++	++

tions C-11 and C-2 and the precipitates which formed were centrifuged in the cold after each addition. When no further precipitation occurred, the supernatants were divided into three portions for testing against the three antigens used. The precipitates were washed in the cold and their nitrogen contents determined in the usual manner. The results are listed in Table VI. It will be seen that absorption with antigen was quite complete in each case and that the serum, following treatment with *Proteus*, no longer contained precipitins for either Fraction C-11 or C-2. Whereas Fractions C-11 and C-2 removed about the same amount of antibody from the serum, the antibody removed was evidently not entirely identical in the two instances.

The sera obtained from rabbits inoculated with *Proteus* OX-19 clearly

contained more antibodies for the two antigenic fractions isolated from this organism than did the antisera prepared with these fractions themselves. Similar observations have been made with the O antigen of the typhoid bacillus isolated from a trichloroacetic acid extract (25).

Test for Agar Contamination—To test for agar contamination, an immunological procedure was devised which required less than 1 mg. of material.

The test was carried out by placing 0.1 cc. portions of a horse antiserum to *Hemophilus influenzae* containing considerable amounts of antiagar precipitins[7] in three sets of micro test-tubes. To one set was added 0.1 cc. of serial dilutions of the agar used in this work in physiological saline, covering the range of 1:1000 (0.1 mg.) to 1:1,000,000. Saline solutions of Fractions C-11 and C-2 in the same concentration range were added to the remaining sets. Controls of normal horse serum were included. The

TABLE VI

Antibody Nitrogen Precipitated from 1 Cc. of Anti-Proteus OX-19 Serum by Successive Additions of Proteus OX-19 and Fractions C-11 and C-2

Antigen added	Antigen N added	N in ppt.	Antibody N pptd.	Supernatant tests with		
				Proteus OX-19	Fraction C-11	Fraction C-2
mg.	*mg.*	*mg.*	*mg.*			
8.00 *Proteus* OX-19	1.932	3.286	1.354	—	—	—
0.197 Fraction C-11	0.010	0.426	0.416	++++	—	++
0.195 " C-2	0.010	0.440	0.430	++++	+	—

tubes were kept stoppered at 37° for 4 hours and overnight in the ice box. Precipitation was observed only in the agar set in all the tubes up to and including the 1:500,000 dilutions; no precipitation, however, occurred in any of the tubes containing the antigens even in the highest concentration (1:1000). To test whether Fractions C-11 and C-2 had any inhibitory effect on the antibody to agar, 0.1 cc. of agar (0.1 mg.) was added to each of the tubes containing the agar antiserum and Fractions C-11 or C-2 after an incubation for 4 hours at 37° and overnight in the ice box. The precipitation of the agar was not impaired. Crude antigen preparations, *viz.* the total trichloroacetic acid extract (Fraction C), were also examined by this procedure. A dilution of Fraction C of 1:4000 gave a weak but significant precipitin reaction. In the subsequent isolation of Fractions C-11 and C-2 from Fraction C, this small impurity is apparently removed.

[7] Placed at our disposal through the kindness of Dr. Hattie E. Alexander of the Department of Pediatrics.

Test for Blood Group Activity—Fractions C-11 and C-2 were found to be devoid of blood group A activity when tested for their ability to inhibit agglutination of human group A red cells by anti-A serum (26).

Studies on Degradation of Antigenic Fractions

The effects of chemical operations, short of total hydrolysis, on the chemical and immunological properties of the two antigenic components of *Proteus* OX-19, were studied in a variety of ways.

Effect of Glacial Acetic Acid on Fractions C-11 and C-2—Weighed portions of Fraction C-11 (102.1 mg.) and of Fraction C-2 (107.9 mg.) were each suspended in 10 cc. of glacial acetic acid and kept, with occasional agitation, for 2 days at room temperature in a nitrogen atmosphere. Following the addition of ether, the insoluble material was filtered off, washed with ether, and dried. The combined ether and acetic acid extracts yielded colorless semicrystalline residues. The material obtained from Fraction C-2 had a neutralization equivalent of 394.

The portions insoluble in a mixture of ether and acetic acid were extracted three times with a total of 10 cc. of water. The fractions soluble in water, recovered from the dialyzed aqueous solutions, and the water-insoluble fractions formed white, fluffy felts, which could both be easily dispersed in physiological saline.

The results of this experiment, summarized in Table VII, demonstrate the far reaching effect of glacial acetic acid. Its most important features were (1) the cleavage of both antigens into water-soluble, water-insoluble, and lipide fractions, accompanied in Fraction C-2 by the loss of cross-reactivity with typhus serum but in Fraction C-11 by an increase in reactivity with the homologous antiserum; (2) the removal of a portion (15 to 18 per cent) of the phosphorus, bound in a labile organic linkage, of which more will be said later; (3) the creation of more reducing groupings in the fragments than were originally present in the intact substances.

Effect of Various Agents on Fraction C-11—A 0.5 per cent solution of Fraction C-11 in pure dry *formamide*, stored in the refrigerator for 2 days, slowly deposited about two-thirds of the starting material as an insoluble precipitate, but neither this fraction nor the material remaining in solution was appreciably different from the original antigen in its reactivity to the homologous antiserum and in its chemical composition. A portion of the total organic phosphorus (14 per cent), however, was not recovered; this occurred in all degradation experiments reported here.

When solutions of Fraction C-11 in 0.1 N *acetic acid* or in 0.05 N *sodium hydroxide* were kept at 100° for 15 minutes, their serological reactivity was destroyed. But the examination of the chemical composition of the fragments (isolated by virtue of their solubility in water and in dilute ethyl

TABLE VII

Degradation of Fractions C-11 and C-2 by Glacial Acetic Acid

Antigen	Fraction	Yield	N	P	N:P	Reducing sugars (as glucose)		Glucos-amine	Precipitation end-points*		
						Without hydroly-sis	After 3 hrs. hydrolysis with N HCl		Fraction C-11 antiserum	Fraction C-2 antiserum	Endemic typhus serum
		per cent	per cent	per cent		per cent	per cent	per cent			
C-11	Starting material	100	5.1	2.5	4.5	2.6	37.5	23.0	1:256,000	1:32,000	No ppt.
	Ether-soluble	3.1		1.2					No ppt.		" "
	Water-soluble	41.5	5.4	2.1	5.7	7.3	55.0	35.1	1:1,024,000		" "
	Water-insoluble	49.4	4.8	2.3	4.6	4.5	34.8	12.1	1:64,000		" "
	Recovery	94.0	90.2	80.4			107	89.6			
C-2	Starting material	100	5.1	2.5	4.5	2.6	25.2	11.2	1:128,000	1:256,000	1:32,000
	Ether-soluble	16.1	0.61	1.3	1.0					No ppt.	No ppt.
	Water-soluble	20.7	4.2	2.8	3.3	10.2	48.3	21.3		1:8000	" "
	Water-insoluble	61.7	6.3	2.1	6.6	5.2	31.5	9.8		1:64,000	" "
	Recovery	98.5	96.0	84.0			117	93.8			

* The antisera were employed in a dilution of 1:10. The highest concentration in which fractions that failed to give precipitation were tested was 1:1000.

alcohol of varying concentrations), following prolonged treatment with these agents, revealed that only slight fractionation into the component parts had taken place.

No cleavage of Fraction C-11 nor loss of serological reactivity was observed when the antigen was treated with 0.5 per cent aqueous *sodium desoxycholate* for 20 hours at 4°.

Freezing in Presence of Ether (27, 28)—When a 1 per cent aqueous solution of Fraction C-2 was extracted nine times in the frozen state with ether by the technique described previously (28), about 4.7 per cent of a lipide fraction, *i.e.* slightly more than one-third of the total lipide contents, was found in the ethereal extract. The aqueous phase, following the complete removal of dissolved ether, was centrifuged at 31,000g for 1 hour, when complete sedimentation had taken place. The aqueous solution of the sediment, centrifuged for 10 minutes at 5000g, yielded a deposit (21 per cent of the starting material) with unchanged reactivity toward typhus serum and a turbid supernatant from which a fraction was recovered (43 per cent of the starting material) that was inactive when tested with typhus serum.

Fraction C-11, subjected to the same operation, yielded practically no lipide. The serologically unchanged material could be recovered following the freezing process.

Serological Stability of Antigens toward Heating in Water and in Alcohol-Ether—For the examination of heat stability 0.1 per cent aqueous solutions of Fractions C-11 and C-2 were kept at 100° for 1 hour. The results included in Table VIII show that, whereas, the reactivities of both fractions toward *Proteus* OX-19 antiserum were diminished only to a small extent, the cross-reactivity of Fraction C-2 with typhus serum was completely destroyed by heat.

The serological stability of both antigenic fractions toward alcohol-ether (1:1) was tested with 5 per cent suspensions of these fractions in the solvent mixture. The suspensions were brought to the boiling point of the solvent (49°), immediately cooled, and kept in the refrigerator overnight. The extraction residues were removed by centrifugation, washed with alcohol-ether, and dried. The extracts yielded small amounts of a lipide mixture (less than 1 per cent from Fraction C-11, 6.3 per cent from Fraction C-2). The serological activities of the extracted antigens are likewise presented in Table VIII. It will be seen that the effect of the partial removal of lipides was very similar to that of heat: the reactivity of Fraction C-2 toward typhus serum was completely abolished.

Since the treatment of Fraction C-2 with alcohol-ether resulted in the loss of its cross-reaction with typhus serum, it appeared of interest to determine whether alcohol-ether extraction would change the serological properties of

the intact organisms. It was found that the extraction of the dry organisms with alcohol-ether (1:1) for 6 days at room temperature effected the removal of 3.6 per cent of lipide material, whereas with ether alone only 0.3 per cent of lipide material was removed. The extraction of *Proteus* OX-19 with these organic solvents appeared to be without significant effect on the agglutinogens present in the organisms since the agglutination end-points of untreated and defatted organisms with *Proteus* antiserum and endemic typhus convalescent serum were found to be identical, at least within the experimental error.

<div align="center">

TABLE VIII

Serological Stability of Fractions C-11 and C-2

</div>

Serum (1:10)	Final antigen dilutions showing precipitation					
	Fraction C-11			Fraction C-2		
	Untreated	Heated	Extracted with alcohol-ether	Untreated	Heated	Extracted with alcohol-ether
Human endemic typhus serum	No ppt.	No ppt.	No ppt.	1:32,000	No ppt.	No ppt.
Proteus OX-19 antiserum	1:64,000	1:16,000	1:32,000	1:64,000	1:16,000	1:64,000

Lipide Components

For extraction of the lipides, 1.09 gm. of Fraction C-11 and 1.01 gm. of Fraction C-2 were each refluxed for 24 hours with 150 cc. of equal volumes of absolute ether and alcohol in a nitrogen atmosphere. The extraction residues were removed by filtration through fine sintered glass funnels, washed with alcohol and ether, and dried. The combined extracts and washings were concentrated in a stream of nitrogen under reduced pressure and the residues dried *in vacuo*. The lipide fractions, representing about 2.8 per cent of Fraction C-11 and about 11.1 per cent of Fraction C-2, were pale yellow and had a crystalline appearance. The test for acetal phosphatides (29) was negative.

The lipides were each dissolved in 1 cc. of ether and the acetone-insoluble fractions precipitated by the addition of 5 cc. of acetone. The combined supernatants and washings yielded the acetone-soluble portions as almost colorless, crystalline residues. The acetone-insoluble lipides were light gray in color, amorphous, and granular, and were for the most part insoluble in alcohol. The figures presented in Table IX indicate that the acetone-insoluble lipide fraction from Fraction C-2 is composed predominantly

(about 64 per cent) of phosphatides containing free amino groups. The analysis of this lipide fraction by the ninhydrin method (31) revealed the absence of combined amino acids. Insufficient amounts of the acetone-insoluble lipide from Fraction C-11 precluded a more detailed analysis of this fraction, but the N and P figures indicate a marked similarity with the corresponding fraction from Fraction C-2.

The lipide fractions soluble in acetone were dissolved in dry ether, the solutions were centrifuged, and the supernatants evaporated to dryness, when almost colorless crystalline residues were obtained. These fractions consisted mainly of free fatty acids. With the customary separation by the lead salt-alcohol procedure (32), the lead salts insoluble in alcohol were washed six times in the centrifuge with small portions of cold ethyl alcohol, until the excess of lead acetate was removed completely, and finally recrystallized from the same solvent. The acetone-soluble lipides (14.8 mg.) from Fraction C-11 yielded 11.5 mg. of crude lead salts and 9.2 mg. of the

TABLE IX

Lipides from Fractions C-11 and C-2

Fraction	Lipide fraction	Proportion of total lipides	N	P	N:P	Amino N (cf. (30))
		per cent	*per cent*	*per cent*		*per cent*
C-11	Acetone-soluble	54.6				
	Acetone-insoluble	42.8	1.78	3.75	1.05	
C-2	Acetone-soluble	59.4				
	Acetone-insoluble	39.8	1.80	3.91	1.02	1.16

recrystallized product melting at 106–107°; those from Fraction C-2 (60.3 mg.) gave 37.3 mg. of crude and 31.6 mg. of purified lead salts which melted at 105–106° and contained Pb 29.6 per cent (calculated for lead palmitate, $(C_{16}H_{31}O_2)_2Pb$, 28.9 per cent).[8] The solid free fatty acids recovered from the lead salts from Fraction C-11 weighed 5.9 mg., melted at 57–57.5°, and had a neutralization equivalent of 256; those from Fraction C-2 weighed 17.3 mg., melted at 57–57.5°, and had a neutralization equivalent of 259. (Required by palmitic acid, $C_{16}H_{32}O_2$, melting point 62.9°, neutralization equivalent 256.4.)

The liquid fatty acid fractions (contaminated with small amounts of neutral fat), recovered from Fractions C-11 and C-2 respectively, weighed 5.5 and 21.2 mg. The color reactions for steroids were negative.

[8] All melting points, reported without correction, were determined by means of an electrically heated stage. Lead myristate melts at 108.7°, lead palmitate at 112.3°, lead stearate at 115.7° (33).

Phosphorylated Constituents

Both antigenic preparations contained 2.1 to 2.5 per cent of phosphorus (Table II), all of which was in organic linkage. The course of the liberation of phosphoric acid, presented in Table X, was followed with 1.0 mg. samples which were heated at 100° with 2 cc. of 1 N HCl in sealed tubes for varying time intervals. The mixtures were cooled, neutralized, filtered, and the inorganic P was determined in the filtrates.

These experiments revealed one interesting fact; namely, the presence in both antigens of a portion of the phosphoric acid in a linkage extremely labile to acids. Within 7 minutes, 18 and 17 per cent of the total P contained in Fractions C-11 and C-2 respectively were hydrolyzed by 1 N HCl

TABLE X

Liberation of Phosphoric Acid from Fractions C-11 and C-2 by Acid Hydrolysis
(N HCl at 100°)

Duration of hydrolysis	Inorganic P liberated			
	Fraction C-11		Fraction C-2	
hrs.	*per cent*	*per cent of total P*	*per cent*	*per cent of total P*
0	0	0	0	0
0.12 (7 min.)	0.46	18	0.42	17
0.5	0.59	24	0.47	19
1	0.69	28	0.59	24
3	0.84	34	0.77	31
7	1.14	46	0.95	38
15	1.48	59	1.20	48
23	1.75	70	1.37	55
35	2.01	81	1.61	66
47	2.17	87	1.78	72
Complete digestion	2.49	100	2.48	100

at 100°. The remainder of the phosphoric acid was apparently attached in a different type of linkage which was broken by acid only very gradually; even after 47 hours the liberation of inorganic P was not complete. The figures presented in Table X demonstrate the similarity in the rates of hydrolysis of the phosphorylated constituents of both antigens.

The inactivity of acid molybdate in catalyzing the dephosphorylation excluded the presence of creatine phosphate (34) or of acyl phosphates (35), nor did the antigens exhibit the behavior toward acid hydrolysis characteristic of phosphoarginine (36).

A preparation of purified beef intestinal phosphatase (37), obtained through the courtesy of Dr. G. Schmidt of the Boston Dispensary, failed to split off any phosphoric acid from Fractions C-11 and C-2 within 18 hours at pH 9.2 and 37°. The same enzyme preparation hydrolyzed β-glycerophosphoric acid to the extent of 94 per cent.

Amino Acid Composition

Qualitative chemical tests, referred to in a preceding section, indicated the presence in both Fractions C-11 and C-2 of a protein or a polypeptide. Tyrosine, tryptophane, and histidine appeared to be absent.

The values presented in Table XI were determined following the hydrolysis of samples with 6 N hydrochloric acid for 16 hours at 100°. Less drastic hydrolytic conditions gave much lower figures for amino N, α-amino acid N, and amino sugar. The neutralized hydrolysates, clarified by centrifugation, served for the estimation of seven amino acids, listed in Table XI, that comprised about 44 per cent of the total α-amino acid N of the two antigen fractions.

TABLE XI

Amino N, α-Amino Acid N, and Amino Acid Contents of Fractions C-11 and C-2

	Fraction C-11			Fraction C-2		
	per cent	*per cent of total N*	*per cent of α-amino acid N*	*per cent*	*per cent of total N*	*per cent of α-amino acid N*
Amino N...............	4.1	81		4.4	86	
α-Amino acid N (*cf.* (31))..................	1.4	27		2.4	47	
Arginine................	0.8	5.1	4.6	1.1	6.9	3.7
Glutamic acid	1.6	3.0	10.9	3.0	5.6	11.9
Isoleucine...............	1.3	2.7	9.9	1.7	3.6	7.6
Leucine.................	0.6	1.3	4.6	1.5	3.2	6.7
Lysine.................	0.3	1.1	2.1	0.5	1.9	2.0
Phenylalanine...........	0.6	1.2	3.6	1.0	1.7	3.5
Proline.................	0.9	2.2	7.8	1.7	4.1	8.6

The values for arginine were determined by the method of Brand and Kassell (38). For the assays of glutamic acid, phenylalanine, and isoleucine, performed by means of *Lactobacillus arabinosus*, we are obliged to Dr. E. Brand and Dr. L. Saidel of this Department. The bacterial growth was measured turbidimetrically (39–41). Lysine, proline, and leucine were determined with the help of the corresponding deficient mutant strains of *Neurospora crassa*. The procedure described for leucine by Ryan and Brand (42) was followed, except for the use of smaller amounts. Sterilized samples of the neutralized hydrolysates and parallel samples of *l*-lysine, *l*-proline, and *l*-leucine, covering a concentration range up to 50 γ in a total volume of 5.0 cc. of modified Fries medium (42), were placed in 10 cc. test-tubes and inoculated with the corresponding mutant strains.[9] The cul-

[9] We are greatly indebted to Dr. F. J. Ryan of the Department of Zoology for his helpful cooperation in these assays.

tures were incubated at 30° for 8 days and the mycelia separated by centrifugation, washed with water, and dried to constant weight at 100°. Standard curves, relating mycelial weights to known quantities of the amino acids, served for the estimation of the contents of the hydrolysates.

Determination of Acetyl Groups

Method—The procedure, based on the principle of isotope dilution, was tested with a specimen of N-acetyl-*d*-glucosamine prepared by synthesis (43). This preparation melted at 206–207° (with decomposition) and contained N (Dumas) 6.29 per cent; calculated for $C_8H_{15}O_6N$ (221.2), N 6.33 per cent. Its rotation (1 per cent aqueous solution, at equilibrium) was $[\alpha]_D^{22} = +39.5°$.

A solution of 102.4 mg. of *N-acetyl-d-glucosamine* (0.46 mM) and of 3.976 mg. of labeled *sodium acetate* (containing 9.8 atom per cent C^{13} excess, 77 atom per cent deuterium excess, corresponding to a molecular weight of 84.5)[10] in 5 cc. of N sulfuric acid was heated in a sealed tube for $2\frac{1}{2}$ hours in a boiling water bath. The cooled hydrolysis mixture was transfered to an all-glass steam distillation apparatus with the aid of 15 cc. of N H_2SO_4 and subjected to steam distillation at constant volume for 30 minutes until 100 cc. of distillate had collected. The distillate was vigorously shaken with 900 mg. of silver carbonate for 5 minutes, and the filtrate was concentrated under reduced pressure to a volume of 10 cc., clarified, and chilled following the addition of 2 volumes of ethyl alcohol and 1 volume of ether. The *silver acetate*, washed with alcohol and ether and dried *in vacuo*, weighed 41.7 mg. (corresponding to a recovery of 48.9 per cent of the total acetic acid present).

For analysis, 20.56 mg. of the silver acetate preparation were burned in a stream of oxygen; the vapors were passed through cupric oxide heated to 600° and then through saturated barium hydroxide. The precipitated barium carbonate was washed and converted to CO_2 which was analyzed for its C^{13} content in the mass spectrometer.[10] The residue of metallic silver remaining after the combustion of the silver acetate was weighed. The customary calculations (44) served for the evaluation of the isotope dilution. The sample of N-acetyl-*d*-glucosamine was found to contain 0.46 mM or 19.9 mg. of acetyl.

$C_2H_3O_2Ag$ (166.9).	Calculated.	Ag 64.7
	Found.	" 64.8, C^{13} excess 0.907 atom %
$C_8H_{15}O_6N$ (221.2).	Calculated.	CH_3CO 19.5
	Found.	" 19.5

[10] We are indebted to Dr. D. Rittenberg for samples of the labeled sodium acetate and for valuable advice concerning the measurements. We also thank Mr. I. Sucher of this Department for carrying out the C^{13} analyses.

Acetyl Content of C-11 and C-2—For the determination of the acetyl content, the antigenic Fractions C-11 and C-2 were first freed of lipides by extraction with alcohol-ether (1:1) as described above. The conditions of acid hydrolysis (N sulfuric acid, 3 hours at 100°) were known to insure the maximum liberation of reducing sugars, the complete deacetylation of N-acetyl amino sugars, and the liberation of possibly present O-acetyl which, in general, is more easily hydrolyzed (45).

For acetyl determination, mixtures of 976 mg. of Fraction C-11 (free of lipides) and 9.136 mg. of the labeled sodium acetate (see above) and of 797 mg. of Fraction C-2 (free of lipides) and 4.757 mg. of labeled sodium acetate were each hydrolyzed with 20 cc. of N H_2SO_4 at 100° in sealed tubes for $2\frac{1}{2}$ hours. The dark brown hydrolysis mixtures were filtered through sintered glass filters, the residues were washed with N H_2SO_4, and the combined filtrates and washings subjected to steam distillation for 30 minutes at constant volume. The distillation residues served for the subsequent isolation of carbohydrates which will be discussed later. The separation of silver acetate, carried out as described in the first portion of this section, yielded 121.6 mg. of silver acetate from Fraction C-11 and 30.8 mg. from Fraction C-2.

$C_2H_3O_2Ag$ (166.9). Calculated.   Ag 64.7
                 Found. Fraction C-11.   " 64.6, C^{13} excess 0.70 atom %
                        " C-2.   " 64.8, "   " 1.21 " %

The acetyl values found were 6.18 per cent for Fraction C-11 (lipide-free) and 6.01 per cent for the intact fraction; 2.16 per cent for Fraction C-2 (lipide-free) and 1.92 per cent for the intact fraction.

Amino Sugar Content

Estimation of N-Acetyl Hexosamine—The Zuckerkandl procedure (43) as modified by Morgan and Elson (46) was employed. The application of this method to polysaccharides is restricted by the fact that the presence of the free aldehyde group in the acetylated amino sugar is required (47). Although the glycosidic linkage in a N-acetylated hexosaminide appears to be broken with greater ease by acid than the N-acetyl bond (48), the values obtained for the N-acetyl hexosamine content of a polysaccharide can have only qualitative significance.

The hydrolysis of the antigenic Fractions C-11 and C-2 with N acid at 100° for 3 hours produced maximum reducing values (compare Table XII), but the hydrolysates contained no N-acetyl hexosamine when examined by quantitative colorimetry. Following a hydrolysis for 30 minutes only, however, when about two-thirds of the maximum reducing values were obtained with both fractions, strong reactions for N-acetyl hexosamine

were found.[11] The values included in Table XII show that about 42 per cent of the total hexosamines of Fraction C-11 (calculated as glucosamine) and 58 per cent of those of Fraction C-2 remained in the acetylated form following this partial hydrolysis.

The acetyl values reported in the preceding section, *viz.* 6.0 and 1.9 per cent in Fractions C-11 and C-2 respectively, would correspond to 25 and 8

TABLE XII

Comparison of Rates of Liberation of Phosphorus, Reducing Sugars, and Amino Sugars by Acid Hydrolysis of Fractions C-11 and C-2

Fraction	Conditions of hydrolysis at 100°		Inorganic P		Reducing sugars (as glucose)	N-Acetylhexos-amine		Hexosamine (as glucosamine)	
	Normality of HCl	Duration							
		hrs.	per cent	per cent of total P	per cent	per cent	per cent of total hexosamine	per cent	per cent of total hexosamine
C-11	1	0	0	0	2.7	0	0	0	0
		0.12	0.46	18	21.2			1.4	6
		0.5	0.59	24	28.7	12	42	2.1	9
		1	0.69	28	33.7			2.7	12
		2			36.0			3.1	13
		3	0.84	34	37.5	0	0	3.4	15
		4			36.3				
	4	8	1.29	52				10.7	48
	6	16	2.47	99	29.2			23.0	100
		24			25.8			21.6	94
C-2	1	0	0	0	2.6	0	0	0.40	4
		0.12	0.42	17	14.1			0.95	9
		0.5	0.47	19	18.4	8	58	1.5	13
		1	0.59	24	21.4			1.7	15
		2			25.2			1.8	16
		3	0.77	31	25.2	0	0	1.8	16
		4			24.9				
	4	8	1.19	48				6.6	59
	6	16	2.45	99	17.8			11.2	100
		24			15.0			10.6	95

per cent of hexosamine if all the acetyl were linked to amino sugar N in these fractions. The actual hexosamine content of different preparations of Fraction C-11 lay between 23 and 24 per cent, that of Fraction C-2 between 10 and 11 per cent (compare Table II). The agreement is close enough for the assumption that all the amino sugar present in the antigens

[11] The colorimetric determinations were carried out in a Klett-Summerson photo-electric colorimeter with a No. 54 filter.

carried acetyl on its amino group. That at least a portion of these amino sugars consisted of d-glucosamine will be shown later.

Estimation of Total Hexosamine—In the colorimetric determination of amino sugars (23, 49) by successive treatment with acetylacetone (50) and p-dimethylaminobenzaldehyde, both the amino and the aldehyde groups have to be free.

The ease with which free amino sugars are produced in the hydrolysis of amino polysaccharides varies considerably. While in several instances complete liberation of the amino sugar has been observed upon brief hydrolysis with N acid, other polysaccharides have proved extremely resistant.

The procedure for the determination of the hexosamine content of the *Proteus* fractions followed, with a number of modifications, the adaptation of the Elson and Morgan method (23) described by Palmer *et al.* (49). The most important deviation from the method prescribed by these authors consisted in the conditions of hydrolysis. The establishment of a detailed hydrolysis curve permitted the definition of conditions leading to the maximum recovery of hexosamine. Hydrolysis of Fractions C-11 and C-2 with 4 N HCl for 8 hours (49) gave rise to only about half of the total hexosamine contained in these fractions (compare Table XII). A much more vigorous hydrolysis proved necessary.

Samples containing 10 to 50 γ of hexosamine were hydrolyzed with 0.5 cc. of 6 N HCl in sealed tubes for 16 hours at 100°. The opened tubes were placed in an ice bath, a small drop of 0.05 per cent phenolphthalein was introduced, and 3 N NaOH was carefully added in small portions until the first indication of alkalinity.[12] The solution was neutralized with a few drops of 0.01 N HCl and diluted to 5 cc. Duplicate 2.0 cc. aliquots were placed in 5 cc. glass-stoppered volumetric flasks (Pyrex), 0.5 cc. of the alkaline acetylacetone reagent (49) was added, and the stoppered flasks were placed upright in a boiling water bath for 30 minutes. Standard samples of glucosamine were always included for comparison. The remainder of the analysis followed the procedure of Palmer *et al.* (49). The samples were read in the Klett-Summerson photoelectric colorimeter with No. 54 filter.

With regard to interference by other substances (*cf.* (23)) some experiments were carried out with mixtures of sugars and amino acids, in the presence and absence of glucosamine. Neither amino acids nor sugars alone interfered with the determination of glucosamine. A mixture of 30 γ of glucose and 20 γ of glycine, however, gave rise to a color equivalent to 2.5 γ of glucosamine. When these quantities were quintupled, a color corresponding to only 4.0 γ of glucosamine developed. A mixture of 150

[12] A slight overneutralization of the well chilled hydrolysate did not affect the results.

γ of glucose and 100 γ of glutamic acid equaled the color given by 3.3 γ of glucosamine. The interference exerted by these substances may, therefore, normally be disregarded unless their proportion to that of the amino sugars is exceptionally unfavorable.

The rates of liberation of phosphorus, reducing sugars, and N-acetyl and free amino sugars in the course of the acid hydrolysis of Fractions C-11 and C-2 are compared in Table XII. These values manifest a remarkable parallelism in the rates of liberation of inorganic P and of hexosamine. *The complete liberation of the organically bound P appeared to be a requisite for the arrival at maximum hexosamine values.* About half of the total amino sugar was liberated as the N-acetyl derivative after a 30 minute hydrolysis with N acid. Hydrolysis for 8 hours with 4 N acid, however, was required for the liberation of the same amount of amino sugar in the free form. It is clear that this apparent discrepancy cannot be due to the resistance of the N-acetyl group to acid hydrolysis, since a 3 hour hydrolysis sufficed for complete deacetylation without a corresponding liberation of free hexosamine; nor can it be due to the destruction of the deacetylated amino sugar, since free glucosamine is stable toward acids (*cf.* (51)). The best explanation, if the parallelism in the liberation of P and of hexosamine is taken into account, consists in the assumption that the *Proteus* antigens contain phosphorylated amino sugars in which, before dephosphorylation (to which they are particularly resistant) has taken place, the condensation with acetylacetone is inhibited.

Carbohydrate Constituents

The results presented in Table XII demonstrate that Fractions C-11 and C-2 contained 14.5 and 14.0 per cent respectively of reducing sugars in addition to hexosamine. The qualitative tests reported above made the presence of significant amounts of sugars other than aldohexoses appear unlikely. The attempt to characterize the component carbohydrates by isolation of suitable derivatives was preceded by orienting experiments on the behavior of the hydrolysis mixtures toward fermentation by bakers' yeast.

Fermentation Experiments—Suspensions of 200 mg. of washed bakers' yeast cells in 1.0 cc. of water were incubated at 37° with 4.0 cc. of an aqueous solution of 1.5 mg. of a known sugar or with 4.0 cc. of a neutralized hydrolysate (3 hours, N HCl) of Fractions C-11 or C-2. Following incubation periods of 15 minutes and of 3 hours, the suspensions were clarified by centrifugation and aliquots were analyzed for remaining reducing sugar by the Hagedorn-Jensen method. The necessary controls with yeast in the absence of substrates were, of course, included. The results, summarized in Table XIII, show that in both Fractions C-11 and C-2 the bulk of the re-

ducing sugars present resisted enzymatic degradation; they probably con-
sisted of galactose and glucosamine. The small portion of fermentable
sugars resembled *d-glucose* most closely in its behavior toward yeast.[13]

Isolation of Mannose and Galactose from Fraction C-11—The acidic hy-
drolysates (3 hours, N H_2SO_4) employed in the acetyl determinations
described above served, after the removal of acetic acid by steam distillation,
for the isolation of sugar derivatives (22) from both Fractions C-11 and C-2.
Following extraction with petroleum ether, which yielded practically no
residue, the aqueous phase was brought to pH 7.1 by the addition of
saturated barium hydroxide with vigorous stirring in the cold. Charcoal
was added and the mixture was filtered through a thin layer of diatomaceous
earth. The filtrate was made slightly acid with glacial acetic acid, con-

TABLE XIII

Fermentation by Yeast

	Substrate		Fermentation	
	Weight		After 15 min.	After 3 hrs.
	mg.		*per cent*	*per cent*
Fraction C-11	9.50 (neutralized hydrolysate con-taining 3.56 mg. reducing sugars, calculated as glucose)		3.9	12.4
" C-2	9.51 (neutralized hydrolysate con-taining 2.40 mg. reducing sugars)		3.3	12.5
d-Glucose	1.5		33.4	98.8
d-Mannose	1.5		3.0	98.4
d-Galactose	1.5		0	3.0
d-Glucosamine	1.5		0.2	11.7

centrated below 30° under reduced pressure, and brought exactly to 100 cc.
The hydrolysate obtained in this manner from Fraction C-11 was found to
contain 284 mg. of reducing sugars (calculated as glucose) which included 197
mg. of hexosamine (calculated as glucosamine, determined by additional
hydrolysis with 6 N HCl for 16 hours).

An aliquot of this hydrolysate (containing 220 mg. of reducing sugars or
68 mg. of non-nitrogenous aldohexoses) was concentrated to 1 cc. under
reduced pressure. The addition of 150 mg. of freshly distilled phenyl-
hydrazine in 25 per cent acetic acid led to the deposition of a small amount
of crystalline material which after five recrystallizations from 60 per cent
ethyl alcohol yielded 0.9 mg. of white prisms melting with decomposition

[13] *d*-Glucosamine is usually found not to be fermented by yeast (*cf. e.g.* (52)). The
reason for the disappearance of a small portion of the amino sugar in the experiment
included in Table XIII cannot be stated.

at 197–198°. The mixture of this substance with an authentic specimen of *d-mannose phenylhydrazone* (m.p. 198–198.5°) melted at 197–198°.

The filtrate from the mannose phenylhydrazone was freed of excess hydrazine by treatment with benzaldehyde in the customary manner. The addition of 150 mg. of freshly distilled α-methylphenylhydrazine in 0.5 cc. of alcohol to the concentrated solution (0.5 cc.) brought about the formation of 43.7 mg. of light brown crystals. Pure *d-galactose α-methylphenylhydrazone* was obtained after several recrystallizations from 30 per cent alcohol as shining plates which weighed 14 mg. and melted with decomposition at 188–189°. No depression of the melting point was observed on admixture of an authentic specimen of this hydrazone.

$C_{13}H_{20}O_5N_2$.   Calculated.   C 54.9, H 7.1, N 9.9
284.3   Found.   " 54.8, " 7.1, " (Dumas) 9.7

Isolation of Mannose and Galactose from Fraction C-2—The hydrolysate of Fraction C-2, prepared as described above for Fraction C-11, contained, in 100 cc. of aqueous solution, 142 mg. of reducing sugars of which 85 mg. were hexosamine, and after concentration yielded 1.6 mg. of an insoluble phenylhydrazone which after two recrystallizations from 60 per cent alcohol gave 0.5 mg. of the white prisms of *d-mannose phenylhydrazone;* its melting point, 197–198° (with decomposition), was not depressed by admixture of a synthetic specimen.

The filtrate furnished 28.6 mg. of a crude α-methylphenylhydrazone from which after six recrystallizations from 30 per cent alcohol 5.1 mg. of pure *d-galactose α-methylphenylhydrazone* were obtained, melting with decomposition at 187°. The melting point was not depressed in a mixture with a genuine sample of this compound.

$C_{13}H_{20}O_5N_2$ (284.3). Calculated, N 9.9; found, (Dumas) 10.1

Isolation of Glucosamine from Fractions C-11 and C-2—Of the several methods for the isolation of the hexosamine (52–54) the preparation of the Schiff base with 2-hydroxynaphthaldehyde (54) was chosen, as this procedure is equally adaptable to glucosamine and chondrosamine.

The filtrates from the methylphenylhydrazones were freed of excess hydrazine by treatment with benzaldehyde and benzoic acid and evaporated to dryness *in vacuo.* The residues (from Fractions C-11 and C-2) were each subjected to an additional hydrolysis with 20 cc. of 6 N HCl for 16 hours at 100°, in order to effect the complete liberation of the amino sugars (compare Table XII). The clear, slightly colored solutions, obtained after treatment of the hydrolysates with norit and filtration through diatomaceous earth, were concentrated and freed of HCl under reduced pressure.

The final solutions (0.5 cc. volume) were adjusted to pH 5.0 by the addi-

tion of $KHCO_3$ and 200 mg. of sodium acetate were added. The solution resulting from Fraction C-11 was treated with a solution of 600 mg. of freshly synthesized (55) 2-hydroxynaphthaldehyde (m.p. 82°) in 4 cc. of methyl alcohol, that from Fraction C-2 with 250 mg. of the same reagent. The mixtures were kept for 3 hours in the dark at room temperature and overnight in the refrigerator, taken to dryness under reduced pressure, extracted three times with 6 cc. portions of chloroform-ether (2:1), dried, and extracted four times with 1 cc. portions of ice-cold water. The aqueous extraction removed considerable amounts of amorphous solids from which no definite compounds could be isolated. The water-insoluble fractions were yellow crystalline substances weighing 76 and 33 mg. from Fractions C-11 and C-2 respectively. Recrystallization from methyl alcohol yielded in both cases pure *2-hydroxynaphthylidene-d-glucosamine*, 38 mg. (from Fraction C-11) and 20 mg. (from Fraction C-2) of yellow plates. The derivative from Fraction C-11 melted with decomposition at 198–200°, that from Fraction C-2 at 200–201°. The mixtures with an authentic specimen of the Schiff base (m.p. 199–200°, with decomposition) melted at 198–201° and 199–201° respectively. The specimens were dextrorotatory; $[\alpha]_{5460}^{27} = +212°$ (at equilibrium; in methyl alcohol). The equilibrium rotation reported in the literature (54) is $[\alpha]_{5461} = +217°$.

$C_{17}H_{19}O_6N$.   Calculated.   C 61.3, H 5.8, N 4.2
333.3   Found.   Fraction C-11.   " 61.1, " 6.0, " (Dumas) 4.1
               "   "   C-2.   " 61.2, " 6.0

DISCUSSION

The studies presented in this paper show that two antigenic fractions, amounting together to somewhat more than 4 per cent of the dry microorganisms, can be prepared from *Proteus* OX-19 either following its disintegration by tryptic digestion or, preferably, by extraction with dilute trichloroacetic acid. The major part of the chemical investigations reported here was carried out with material isolated from the trichloroacetic acid extracts. The outstanding immunological properties of *Proteus* OX-19 were retained in these extracts: they gave specific precipitation with antisera to *Proteus* OX-19 and with convalescent typhus sera.

The separation of the two antigenic fractions was based on the large particle size of the substance endowed with specificity to both *Proteus* and typhus sera (designated Fraction C-2) which rendered it sedimentable at a high centrifugal speed, whereas the other antigen (designated Fraction C-11), which possessed *Proteus* specificity only, remained in solution. Both substances were antigenic in the rabbit; their immunological properties are discussed in the experimental part. The course of the reaction, followed by quantitative methods, between the antigens and their rabbit antisera,

especially with respect to anti-Fraction C-11, was that of a typical antigen-antibody reaction (24). The sensitive serological test for the detection of agar impurities in the bacterial antigens, described in this connection, may be of general interest.

The antigenic fractions were electrophoretically homogeneous complexes composed of a lipide, a polysaccharide, and a protein portion. They obviously belong to the extensively studied series of somatic antigen complexes from Gram-negative bacilli ((16, 56–60); *cf.* also (4), p. 222). The structural function within the bacterial cell of these complicated substances is unknown; some of these antigens are known to occur as aggregates of large particle weight, sedimentable at high centrifugal speeds (58, 61).

The two antigenic fractions of *Proteus* OX-19 were strikingly similar in the chemical nature of their components, but there were several important quantitative differences. Perhaps the most important distinction was that Fraction C-2, the particulate antigen exhibiting cross-reaction with typhus sera, contained about 4 times as much lipide as the *Proteus*-specific Fraction C-11. The lipides possibly serve to maintain the large particle size of Fraction C-2 in a manner similar to their rôle in supporting the macromolecular structure of the thromboplastic protein (28, 62). It is remarkable that the partial removal of the lipides from Fraction C-2 under relatively mild conditions results in the destruction of its typhus specificity without impairment of its reactivity with *Proteus* antiserum. Attempts to reconstitute the typhus reactivity by recombining the lipide portion with the rest of the antigen were unsuccessful. The degree to which the two *Proteus* antigens are related to each other cannot yet be decided; they conceivably represent two different states of·polymerization of a basically identical chemical unit.[14] In what manner the large particle size and the high lipide content of Fraction C-2 contribute to its typhus specificity is unknown.

The cross-reactivity with human convalescent typhus serum of Fraction C-2 is completely destroyed by heat, while its reactivity toward *Proteus* OX-19 antiserum is but slightly impaired. This finding, which was made repeatedly with several preparations of Fraction C-2 as well as with the comparable, although less highly purified, Fraction T-2 from a tryptic digest, appears to contradict previous claims (12, 13) concerning the relative stabilities of the *Proteus* OX-19 antigens. The bacterial products used in those stability tests lacked chemical characterization almost completely; so that no comparison with the substances discussed in this

---

[14] It may be of interest to recall that the extraction of a Gram-positive acid-fast organism, the avian tubercle bacillus, with 0.1 N trichloroacetic acid yields a non-nitrogenous macromolecular polyglucosan together with a very similar polysaccharide of lower molecular weight (63).

paper is possible. The experiments on the disintegration of the antigens by various means, a selection of which is included in the experimental part, emphasize the resistance offered by these complexes to cleavage into their protein and polysaccharide moieties. Treatment with glacial acetic acid appeared to be the most promising method.

The study of the constituents of the antigenic complexes requires, with a few exceptions, no extended comment. As mentioned before, the bacterial fractions were compounds between lipides (containing a high proportion of free fatty acids) and phosphorylated polysaccharide-protein complexes. About one-half of the amino acid nitrogen of the protein moiety was. accounted for by the quantitative estimation of seven amino acids; aromatic amino acids seemed to be absent. The polysaccharide portion was found to be composed of N-acetyl-d-glucosamine, which formed the bulk of the carbohydrates, together with d-galactose and very small amounts of d-mannose. Acetylglucosamine and galactose are often encountered as components of heterogenetic antigens (cf. (4), p. 232). Fermentation experiments indicated the presence of a small amount of glucose; the isolation of this sugar, difficult under the experimental conditions, was not attempted.

Attention should be drawn to two features connected with the presence of phosphorus in the antigenic fractions. One has regard to the existence of a portion of the organically bound phosphoric acid in a linkage that proved extremely labile to acid. The other noteworthy feature relates to the possible occurrence in the antigens of phosphorylated amino sugar. A comparative study of the rates of liberation of various components in the course of the acid hydrolysis of the antigens revealed that maximum values for hexosamine could not be obtained before all the organically bound P had been liberated. An extremely long hydrolysis period with 6 N HCl was found necessary. The conclusions from these results were drawn before in this paper, but it should be pointed out that other instances have been recorded, although not expressly noted, in the literature in which a similar situation seems to prevail. For example, the ease with which the amino sugars are liberated from the capsular polysaccharide of pneumococcus type XIV (64) and from the blood group A-specific substances (47, 64), all phosphorus-free compounds, could be contrasted with the refractory behavior of the C and F polysaccharides of pneumococcus type I (65), containing about 4.5 per cent of P, in which, it would seem, only a portion of their amino sugar was liberated under the hydrolysis conditions employed. The observations on the *Proteus* antigens, presented here, emphasize the importance of establishing complete hydrolysis curves with the aid of which the maximum recoveries of the various constituents can be correlated.

It is not yet possible to decide to what extent the experiments outlined here bear on the general problem of the chemistry of heterogenetic antigens. However, inasmuch as Fraction C-2 from *Proteus* OX-19, which exhibits cross-reactivity with convalescent typhus sera, appears to be the *Proteus* agglutinogen involved in the Weil-Felix reaction (1-3), it will be of great interest to compare its properties with those of the *Rickettsia* fraction responsible for the production of agglutinins for *Proteus* OX-19. The treatment of human convalescent typhus serum with Fraction C-2 results in the removal of the Weil-Felix agglutinins. Moreover, rabbit antiserum to Fraction C-2 is a potent agglutinating serum for *Rickettsia prowazeki*. It therefore can hardly be doubted that some relationship exists between the heterogenetic factors of *Proteus* and of *Rickettsia*, but whether this affinity is founded on composition, size, organization, or other properties cannot be discussed before more information on the chemistry of rickettsial constituents has become available.

SUMMARY

Two antigens from *Proteus* OX-19 , an organism known to be agglutinated by typhus convalescent sera, have been prepared and characterized in a variety of ways. The extraction of the microorganisms with trichloroacetic acid (or, less effectively, their digestion with crystalline trypsin) yields two antigenic fractions, different in immunological specificity and in particle size, but rather similar in chemical composition. A heavy particulate fraction (C-2) sedimentable at high centrifugal speeds, is endowed with specificity to typhus sera as well as to *Proteus* antisera; a lighter fraction (C-11) possesses *Proteus* specificity only. Both fractions, which are homogeneous electrophoretically, are phosphorylated lipocarbohydrate-protein complexes. The typhus-reactive Fraction C-2 loses its precipitability by typhus sera when heated or freed of lipides.

The study of the chemical composition of the antigens revealed them as complexes consisting of (*a*) lipides (with a high porportion of free fatty acids), (*b*) a protein (including arginine, lysine, glutamic acid, leucine, iso-leucine, proline, and phenylalanine, but probably free of histidine, tyrosine, and tryptophane), (*c*) a polysaccharide (containing N-acetylglucosamine, mannose, galactose, and perhaps glucose). Part of the organically bound phosphorus is present in the form of an extremely acid-labile linkage.

In connection with the presentation of a modified method for the estima-

tion of amino sugar, the evidence for the occurrence in these antigens of N-acetylglucosamine containing phosphoric acid in ester linkage is discussed.

A method for the quantitative determination of acetyl groups by means of isotope dilution and a sensitive serological test for the detection of agar impurities are likewise presented.

BIBLIOGRAPHY

1. Weil, E., and Felix, A., *Wien. med. Wochschr.*, **29**, 33 (1916).
2. Felix, A., *Brit. Med. J.*, **2**, 597 (1942).
3. Felix, A., *Tr. Roy. Soc. Trop. Med. and Hyg.*, **37**, 321 (1944).
4. Landsteiner, K., The specificity of serological reactions, Cambridge, revised edition (1945).
5. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **154**, 691 (1944).
6. Przesmycki, F., *Compt. rend. Soc. biol.*, **95**, 744 (1926).
7. Furth, J., and Landsteiner, K., *J. Exp. Med.*, **47**, 171 (1928).
8. Lim, C. E., and Kurotchkin, T. J., *Nat. Med. J. China*, **15**, 6 (1929).
9. Meisel, H., and Mikulaszek, E., *Compt. rend. Soc. biol.*, **114**, 364 (1933).
10. Kemp, H. A., and Cain, J. C., *Proc. Soc. Exp. Biol. and Med.*, **32**, 496 (1934).
11. Otto, R., Wissensch. Woche zu Frankfurt-am-Main, Leipzig, **3**, 95 (1935).
12. White, P. B., *Brit. J. Exp. Path.*, **14**, 145 (1933).
13. Castañeda, R., and Zia, S., *J. Exp. Med.*, **58**, 55 (1933).
14. Castañeda, R., *J. Exp. Med.*, **60**, 119 (1934).
15. Castañeda, R., *J. Exp. Med.*, **62**, 289 (1935).
16. Mesrobeanu, L., *Arch. roumaines path. exp. et microbiol.*, **9**, 1 (1936).
17. Ciuca, M., Mesrobeanu, L., Badenski, A., and Munteanu, G., *Compt. rend. Soc. biol.*, **127**, 1414 (1938).
18. Ciuca, M., and Mesrobeanu, L., *Chem. Abstr.*, **38**, 2690 (1944).
19. McFarland, J., *J. Am. Med. Assn.*, **49**, 1176 (1907).
20. King, E. J., *Biochem. J.*, **26**, 292 (1932).
21. McCance, R. A., *Biochem. J.*, **20**, 1111 (1926).
22. van der Haar, A. W., Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin (1920).
23. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).
24. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **65**, 647 (1937).
25. Hornus, G., and Grabar, P., *Ann. Inst. Pasteur*, **66**, 136 (1941).
26. Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, **82**, 297 (1945).
27. McFarlane, A. S., *Nature*, **149**, 439 (1942).
28. Chargaff, E., and Bendich, A., *Science*, **99**, 147 (1944).
29. Feulgen, R., and Grünberg, H., *Z. physiol. Chem.*, **257**, 161 (1939).
30. Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 203 (1934).
31. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
32. Twitchell, E., *J. Ind. and Eng. Chem.*, **13**, 806 (1921).
33. Jacobson, C. A., and Holmes, A. H., *J. Biol. Chem.*, **25**, 29 (1916).
34. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
35. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
36. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **196**, 49 (1928).
37. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).
38. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 359 (1942).

39. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511 (1943).
40. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
41. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, **155**, 591 (1944).
42. Ryan, F. J., and Brand, E., *J. Biol. Chem.*, **154**, 161 (1944).
43. Zuckerkandl, F., and Messiner-Klebermass, L., *Biochem. Z.*, **236**, 19 (1931).
44. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
45. Pregl, F., and Roth, H., Die quantitative organische Mikroanalyse, Berlin, 4th edition, 240 (1935).
46. Morgan, W. T. J., and Elson, L. A., *Biochem. J.*, **28**, 988 (1934).
47. Morgan, W. T. J., and King, H. K., *Biochem. J.*, **37**, 640 (1943).
48. Moggridge, R. C. G., and Neuberger, A., *J. Chem. Soc.*, 745 (1938).
49. Palmer, J. W., Smyth, E. M., and Meyer, K., *J. Biol. Chem.*, **119**, 491 (1937).
50. Pauly, H., and Ludwig, E., *Z. physiol. Chem.*, **121**, 170 (1922).
51. Fischer, E., and Zach, K., *Ber. chem. Ges.*, **44**, 132 (1911).
52. Morgan, W. T. J., *Helv. chim. acta*, **21**, 469 (1938).
53. Chargaff, E., and Bovarnick, M., *J. Biol. Chem.*, **118**, 421 (1937).
54. Jolles, Z. E., and Morgan, W. T. J., *Biochem. J.*, **34**, 1183 (1940).
55. Duff, J. C., and Bills, E. J., *J. Chem. Soc.*, 1305 (1934).
56. Boivin, A., Mesorbeanu, L., and Mesorbeanu, I., *Compt. rend. Soc. Biol.*, **114**, 307 (1933).
57. Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, **15**, 113 (1934).
58. Miles, A. A., and Pirie, N. W., *Brit. J. Exp. Path.*, **20**, 83, 109, 278 (1939); *Biochem. J.*, **33**, 1709, 1716 (1939).
59. Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, **34**, 169 (1940); **35**, 1140 (1941).
60. Freeman, G. G., and Anderson, T. H., *Biochem. J.*, **35**, 564 (1941).
61. Boivin, A., *Compt. rend. Acad.*, **209**, 416 (1939).
62. Chargaff, E., Bendich, A., and Cohen, S. S., *J. Biol. Chem.*, **156**, 161 (1944).
63. Chargaff, E., and Moore, D. H., *J. Biol. Chem.*, **155**, 493 (1944).
64. Goebel, W. F., Beeson, P. B., and Hoagland, C. L., *J. Biol. Chem.*, **129**, 455 (1939).
65. Goebel, W. F., Shedlovsky, T., Lavin, G. I., and Adams, M. H., *J. Biol. Chem.*, **148**, 1 (1943).

COLORIMETRIC DETERMINATION OF METHIONINE IN PROTEINS AND FOODS

By MILLARD J. HORN, D. BREESE JONES, AND AMOS E. BLUM

(From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington)

Most of the earlier methods for the quantitative determination of amino acids are applicable only to purified proteins. Interfering reactions with non-protein substances, particularly carbohydrates, preclude their use for determining amino acids in foods. Great interest has recently developed in the amino acid content of foods, particularly the so called nutritionally essential amino acids. Methods for their determination in foods, therefore, assume increasing importance.

McCarthy and Sullivan (1) described a colorimetric method for the determination of methionine in isolated proteins. This method was later modified by Hess and Sullivan (2). When applying their method to protein foods, we encountered difficulties in its general application. The present paper describes procedures whereby these difficulties have been overcome. Closely agreeing results have been obtained with acid hydrolysates and papain digests of the same proteins and foods. The values are also in close agreement with those obtained for the same materials when determined by a microbiological method.[1]

EXPERIMENTAL

Effect of Temperature on Color Development—The colorimetric method of McCarthy and Sullivan includes the following procedure: To the protein hydrolysates, made strongly alkaline with sodium hydroxide, is added 1 cc. of 1 per cent solution of glycine followed immediately by 0.3 cc. of 10 per cent sodium nitroprusside solution. The mixture is heated for 10 minutes at 35–50°, and then strongly acidified by addition of a mixture of hydrochloric and phosphoric acids. The methionine is determined colorimetrically. The authors state that less color was developed when the temperature was maintained below 35°. The glycine was added to inhibit color formation with other amino acids. Hess and Sullivan subsequently modified the procedure by making the hydrolysates less strongly alkaline, and by using 20 per cent hydrochloric acid instead of the mixture of hydrochloric and phosphoric acids.

---

[1] The microbiological method is described in the paper immediately following.

When applying the procedure to protein material containing carbohydrate, we observed that greenish blue colors frequently developed instead of the red color characteristic for methionine. This difficulty was avoided by carrying out the reaction at room temperature instead of at 35–50°, and by using phosphoric acid instead of hydrochloric acid. That the same color intensity is developed by allowing the solution to stand for 10 minutes at room temperature as by heating it at 35–50° was demonstrated by the following series of tests: 0.1 cc. of a 10 per cent solution of nitroprusside was added to aqueous solutions of methionine at concentrations ranging from 0.0 to 1.0 mg. Readings made with an Evelyn colorimeter showed practically the same color transmissions after the solutions had been heated for 10 minutes at 50° as when they were allowed to remain for the same

TABLE I

Effect of Temperature on Color Transmission

Methionine	Transmission*	
	Room temperature	Heated at 50°
mg.		
0.0	81	81
0.2	63	64
0.4	51	50
0.6	41	42
0.8	31	32
1.0	25.5	26

* These figures, on a scale of 100, show relative transmissions, the transmission with distilled water being set at 100.

time at room temperature (Table I). It was also found that phosphoric acid could be substituted for hydrochloric acid.

Effect of Glycine on Color Intensity—The presence of glycine, added or already in the hydrolysate, reduced color intensity. With the same amount of methionine, decreasing intensities of color were obtained by adding increasing amounts of glycine until the red color was discharged. This effect arises from the difference in the velocity of reaction between methionine and nitroprusside, and between glycine and nitroprusside. Consequently, the glycine originally present in a hydrolysate causes a decrease in color intensity as does also the glycine added to reduce any extraneous colors from other amino acids. In order to provide enough nitroprusside to react with the methionine and still have enough glycine to eliminate extraneous colors, it was found necessary to add 0.1 cc. of 10 per cent sodium nitroprusside solution and 2 cc. of 3 per cent glycine solution. 0.2 cc. of nitroprusside required 4 cc. of glycine, and 0.3 cc. of nitroprusside

required 6 cc. of glycine. Table II shows a direct relationship between the nitroprusside and glycine. For example, 0.6 mg. of methionine gave a transmission reading of 70 when the amounts of nitroprusside and glycine were increased proportionally.

Maximum transmission readings could not be obtained when the glycine was added immediately after addition of the nitroprusside. It was necessary to allow 10 minutes for the nitroprusside to react before adding the

TABLE II

Effect of Glycine on Color Transmission When Amounts of Nitroprusside Remain Constant (Amount of Methionine Used, 0.6 Mg.)

Glycine (3 per cent solution)	Nitroprusside (10 per cent solution)		
	0.1 cc.	0.2 cc.	0.3 cc.
cc.			
1	60	50	42
2	70	62	53
3	75	67	60
4	80	70	67
6		81	70

TABLE III

Effect on Color Transmission of Delaying Addition of Glycine after Addition of Nitroprusside

Methionine used	Transmission	
	Glycine added immediately	Glycine addition delayed 10 min.
mg.		
0.0	92.0	84.0
0.2	84.5	70.0
0.4	77.0	59.5
0.6	70.0	50.0
0.8	64.0	42.0
1.0	57.5	35.5

glycine (Table III). A further delay of 5 to 10 minutes did not affect the results; neither did the addition of more than 2 cc. of glycine as it does in the McCarthy and Sullivan procedure.

Effect of Other Amino Acids—All of the amino acids in a protein hydrolysate participate by contributing some color to the blank, and the addition of glycine does not remove all of this color. Were no methionine present, the hydrolysates of different proteins would doubtless give varying values for the blank readings. With the object of reducing the error from this source, use was made, for the preparation of a standard curve, of a solution

of nineteen amino acids (methionine excluded) in proportions similar to those in 100 cc. of a hydrolysate of 1 gm. of casein. The concentrations of the amino acids in this solution (Solution A) are given in Table IV. A 2 cc. quantity of the solution is comparable to 2 cc. of the protein hydrolysates used in the analyses here reported.

TABLE IV

Composition of Amino Acid Solution A (Mg. per 100 Cc.)

Alanine	20	Leucine	100
Arginine	48	Lysine	60
Aspartic acid	60	Norleucine	30
Cystine	10*	Phenylalanine	40
Glycine	5	Proline	80
Glutamic acid	210	Serine	60
Histidine	30	Threonine	45
Hydroxyproline	10	Tryptophane	12
Isoleucine	50	Tyrosine	65
		Valine	60

* A relatively high proportion of cystine was added, since casein contains less of this amino acid than most proteins.

TABLE V

Effect of Amino Acids Other Than Methionine on Color Transmission

Methionine used	Transmission	
	Methionine alone	Methionine + Solution A
mg.		
0.0	97	84
0.2	77	70
0.4	63	59.5
0.6	52.5	50
0.8	43	42
1.0	35.5	35.5

Table V shows the effect on color transmission of the amino acids in Solution A with increasing amounts of methionine as compared with that observed with the same amounts of methionine alone. The greatest effect occurred at low concentrations of methionine. With concentrations of methionine above 0.6 mg., the transmissions were practically the same.

The amount of glycine in Solution A is small compared with that added during analysis and exerts very little effect. Fortunately, most proteins are low in glycine, and, with the method for methionine determination described here, the glycine would not cause appreciable error.

This method, however, cannot be applied to proteins containing a relatively high content of glycine and a low content of methionine. An acid hydrolysate of gelatin containing 25 per cent of glycine was found to make no contribution to the blank. Assuming that the glycine has to be free in the hydrolysate in order to produce the reducing effect, a papain digest of gelatin, in which the amino acids are in peptide union, should offer little or no difficulty. In fact, a papain digest of gelatin gave values for methionine agreeing closely with those obtained by the microbiological method.

Preparation of Standard Curve—A standard methionine solution was prepared by dissolving 100 mg. of *dl*-methionine in 5 cc. of 20 per cent hydrochloric acid and diluting this solution to 100 cc. with distilled water. Into one of two Evelyn colorimeter tubes were introduced 2 cc. of Solution A, 3 cc. of water, 1 cc. of 5 N sodium hydroxide, and 0.1 cc. of 10 per cent sodium nitroprusside solution. To the other tube were added 2 cc. of Solution A, 1 cc. of the standard methionine solution, 2 cc. of water, 1 cc. of sodium hydroxide, and 0.1 cc. of nitroprusside. After standing for 10 minutes with frequent shaking, 2 cc. of 3 per cent glycine were added to each tube. The mixtures were allowed to stand for 10 minutes with frequent shaking. 2 cc. of concentrated orthophosphoric acid were then added to each tube slowly with constant agitation. After 5 minutes, the tubes were read in an Evelyn colorimeter provided with a No. 540 filter. A tube containing 10 cc. of distilled water was set at 100. Readings were taken for methionine at concentrations ranging from 0.1 to 1.0 mg. The logarithm of the readings plotted against concentration gave a straight line.

Determination of Methionine in Proteins—The protein to be analyzed is first subjected either to acid hydrolysis or to digestion with papain. Essentially the same methionine values were obtained by both methods.

The acid hydrolysis is accomplished by refluxing 1 gm. of the protein for 18 hours with 25 cc. of 20 per cent hydrochloric acid. After the hydrolysate is concentrated to a volume of 5 cc., it is heated to boiling with a small quantity of norit. The mixture is filtered by suction and the residue thoroughly washed on the filter with hot water. The combined filtrate and washings are then made up to a volume of 100 cc. with distilled water.

The papain digestion is carried out as described in a previous paper (3), with the exception that before the digest is made up to volume nitrogen is passed through the solution for 15 minutes to remove hydrocyanic acid. This step is essential; otherwise the cyanide will later reduce the intensity of the methionine color.

For determination of methionine, 2 cc. duplicate samples of the acid hydrolysate, or papain digest, are pipetted into colorimeter tubes. To each tube are added 3 cc. of distilled water and 1 cc. of 5 N sodium hydroxide. To one of the tubes is added 0.1 cc. of nitroprusside. The

other tube is used for blank determination. The procedure is continued as described above for preparing the standard curve.

Recovery Experiments—The accuracy of the method was checked by recovery of the total amount of methionine in an acid hydrolysate of arachin to which known amounts of pure methionine had been added, the methionine content of the hydrolysate having been previously determined. The results are given in Table VI.

The methionine content of a number of proteins and foods determined by the procedures described is given in Table VII. For comparison, there are included values for the same materials determined by a microbiological method.[1]

The proteins analyzed, with the exception of gelatin, were samples previously prepared by methods which have been described: arachin, conarachin, and total peanut globulins (22), cottonseed globulin (23),

TABLE VI

Recovery of Methionine Added to Hydrolysate of Arachin (Methionine in Arachin Hydrolysate, 0.07 Mg.)

Methionine added	Total methionine	Methionine found	Recovery
mg.	*mg.*	*mg.*	*per cent*
0.2	0.27	0.26	96
0.4	0.47	0.48	102
0.6	0.67	0.68	101
0.8	0.87	0.87	100
1.0	1.07	1.05	98

coconut globulin (24), edestin (25), ovalbumin (25), glycinin (26), lactalbumin (27), phaseolin (28), ox muscle (25), wheat bran globulin (29), zein (23), and casein (30). With one exception, all of the foods analyzed were commercial products. The Brazil nut meal was prepared from nuts, ground and defatted in the laboratory. The results obtained by the three procedures for the same materials are in close agreement. They also agree well with those recently reported by others using microbiological methods. Earlier values recorded in the literature for several of the isolated proteins as determined by chemical methods are higher than those here reported.

SUMMARY

1. A colorimetric method is described for the quantitative determination of methionine in isolated proteins and foods.

2. The method is applicable to acid hydrolysates and papain digests of proteins and foods.

3. The methionine contents found for some thirty proteins and foods

TABLE VII

Methionine Content of Various Proteins and Foods (Percentages Calculated on Basis of Ash- and Moisture-Free Material)

Material	N	Methionine			Values from literature
		Colorimetric determinations		Microbiological determinations	
		Acid hydrolysates	Papain digests		
	per cent	*per cent*	*per cent*	*per cent*	
Arachin..................	18.30	0.31	0.35	0.24	0.54 (4), 0.67 (5), 0.77 (6), 0.57 (7)
Casein...................	16.07	2.60	2.58	2.58	2.94–3.64 (1), 2.85 (8), 3.50 (9), 3.12 (7), 3.50 (10), 2.6 (11)
Conarachin..............	18.20	2.24	2.24	2.13	2.12 (5)
Cottonseed globulins.....	18.00	1.45	1.45	1.40	1.7–3.2 (12)*
Coconut globulin.........	17.42	2.12	2.11	2.02	2.05 (13)
Edestin..................	18.55	2.14	2.14	2.12	2.07 (13), 2.3 (14), 2.52 (15), 2.1 (1), 2.30 (7)
Ovalbumin (crystalline)..	15.98	4.48	4.50	4.43	5.10 (9), 5.22 (16), 3.76–4.58 (8), 4.1 (11)
Gelatin (Bacto)..........	18.32		0.76	0.78	0.97 (13), 0.81 (7), 0.59 (11)
Glycinin.................	17.30	1.20	1.20	1.15	1.84 (4)
Lactalbumin..............	15.39	1.71	1.71	1.62	2.98 (7), 2.62 (9), 2.80 (16), 2.4 (8)
Peanut, total globulins...	18.01	1.11	1.20	1.00	
Phaseolin (navy bean)....	16.07	1.21	1.26	1.12	
Ox muscle................	16.00	2.75	2.66	2.75	3.66 (4), 3.3 (10), 3.17 (17), 3.21 (7)
Wheat bran globulin......	17.76	1.21	1.21	1.04	
Zein.....................	16.00	1.62	1.60	1.41	2.32 (18), 1.68 (8)
Barley, pearled...........	1.86	0.10	0.13	0.12	0.13 (19)
Brazil nut meal..........	9.03	2.72	2.88	2.78	
Corn, whole yellow.......	2.22	0.21	0.28	0.20	0.30 (19)
" germ, defatted......	3.93	0.36	0.37	0.35	0.57 (20)
Cottonseed flour.........	10.36	0.88	0.88	0.85	0.81 (19), 1.04 (20)
Egg, whole, dried........	8.11	1.55	1.57	1.39	
Milk, dry, skim..........	6.57	0.89	0.85	0.89	0.87 (11), 0.93 (19), 0.87 (21)
Oatmeal.................	2.73	0.20	0.19	0.18	0.20 (19), 0.39 (20)
Peanut flour.............	10.15	0.58	0.58	0.52	0.57 (20)
Peas, black-eyed.........	4.15	0.26	0.27	0.25	
Rice, white..............	1.26	0.09	0.08	0.11	0.27 (20)
Rye, whole...............	1.98	0.13	0.14	0.14	0.15 (11), 0.29 (20)
Wheat, whole............	3.07	0.23	0.24	0.19	0.23 (11), 0.23 (19)
" germ, defatted.....	6.50	0.56	0.50	0.51	0.81 (20)
Yeast, dried, brewers'....	7.71	0.54	0.56	0.53	0.66 (11), 0.96 (20)

* Analyses on different globulin fractions.

determined chemically on both acid hydrolysates and papain digests are in close agreement. They also agree with values found for the same proteins by a microbiological method.

BIBLIOGRAPHY

1. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
2. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **151**, 635 (1943).
3. Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **157**, 153 (1945).
4. Baernstein, H. D., *J. Biol. Chem.*, **97**, 663 (1932).
5. Brown, W. L., *J. Biol. Chem.*, **142**, 299 (1942).
6. Bennett, M. A., *Biochem. J.*, **33**, 885 (1939).
7. Beach, E. F., and Teague, D. M., *J. Biol. Chem.*, **142**, 277 (1942).
8. Lavine, T. F., *J. Biol. Chem.*, **151**, 281 (1943).
9. Baernstein, H. D., *J. Biol. Chem.*, **106**, 451 (1934).
10. Block, R. J., and Bolling, D., *J. Am. Dietet. Assn.*, **20**, 69 (1944).
11. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
12. Fontaine, T. D., Olcott, H. S., and Lowy, A., *Ind. and Eng. Chem., Anal. Ed.*, **34**, 116 (1942).
13. Baernstein, H. D., *J. Biol. Chem.*, **97**, 669 (1932).
14. Bailey, K., *Biochem. J.*, **31**, 1396 (1937).
15. Beach, E. F., Erickson, B. N., Bernstein, S. S., Williams, H. H., and Macy, I. G., *J. Biol. Chem.*, **128**, 339 (1939).
16. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 145 (1938).
17. Beach, E. F., Munks, B., and Robinson, A., *J. Biol. Chem.*, **148**, 431 (1943).
18. Baernstein, H. D., *J. Biol. Chem.*, **115**, 25 (1936).
19. Baumgarten, W., Mather, A. N., and Stone, L., *Cereal Chem.*, **23**, 135 (1946).
20. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield (1945).
21. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **10**, 55 (1946).
22. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, **28**, 77 (1916–17).
23. Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, **62**, 183 (1924–25).
24. Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, **37**, 149 (1919).
25. Jones, D. B., and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).
26. Csonka, F. A., and Jones, D. B., *J. Agr. Res.*, **46**, 51 (1933).
27. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, **48**, 347 (1921).
28. Waterman, H. C., Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, **55**, 93 (1923).
29. Jones, D. B., and Gersdorff, C. E. F., *J. Biol. Chem.*, **58**, 117 (1923–24).
30. Van Slyke, L. L., and Baker, J. C., *New York State Agr. Exp. Sta., Tech. Bull.* **65** (1918).

MICROBIOLOGICAL DETERMINATION OF METHIONINE IN PROTEINS AND FOODS

By MILLARD J. HORN, D. BREESE JONES, AND AMOS E. BLUM

(*From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington*)

Kuiken and coworkers (1) reported that methionine is not essential for the growth of *Lactobacillus arabinosus* 17-5. Later, Hegsted (2), working with the same organism, found that growth failed when methionine was not present in the medium. He called attention to the apparent discrepancy in the results obtained in the two laboratories and stated that it was probably due to the use of different strains of the organism. Hutchings and Peterson (3) obtained good growth of *Lactobacillus casei* without methionine, but found that it was essential for maximum growth. Shankman, Dunn, and Rubin (4) recommend *Lactobacillus casei* as most satisfactory for the determination of arginine, glutamic acid, leucine, phenylalanine, tryptophane, tyrosine, and valine, but they do not include methionine. Recently, Dunn and coworkers (5) found that methionine can be assayed with *Leuconostoc mesenteroides* P-60. Stokes *et al.* (6) employed *Streptococcus faecalis* for the determination of methionine in several proteins and foods.

EXPERIMENTAL

Lactobacillus arabinosus 17-5 was used in the determinations described in this paper.[1]

Basal Medium—The basal medium (Table I) contained nineteen amino acids in approximately the same quantities of the active isomers as they occur in 1 liter of an acid hydrolysate of 2 gm. of casein, supplemented with cystine and tryptophane in the amounts indicated. 5 cc. of this medium, diluted to 10 cc. with water and incubated for 72 hours, gave the same maximum titration (10 cc.) with 0.1 N sodium hydroxide as a similar medium in which the amino acids had been replaced by an acid hydrolysate of casein supplemented with cystine and tryptophane. 10 cc. of this hydrolysate represented 10 mg. of casein.

Without methionine present, this medium gave a blank titration value of 1.5 cc. of 0.1 N lactic acid after 72 hours of incubation. After addition

---

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

of methionine in the quantity indicated in Table I the medium gave the original maximum titration value of 10 cc. Increasing the quantities of the other amino acids to represent their proportions in 20 mg. of casein did not cause an increase in the titration blank. Addition of methionine to this mixture gave again the maximum titration of 10 cc.

Inoculum—Stab stock cultures of *Lactobacillus arabinosus* were carried in a medium having the following composition: 1 gm. of glucose, 1 gm. of

TABLE I

Composition of Basal Medium

Glucose	20 gm.	*dl*-Alanine	80	mg.
Sodium acetate (anhydrous).	12 "	*l*(+)-Arginine	96	"
Salts A		*dl*-Aspartic acid	240	"
K₂HPO₄	1 "	*dl*-Glutamic acid + H₂O	940	"
KH₂PO₄	1 "	Glycine	400	"
Salts B		*l*(−)-Histidine hydrochloride		
MgSO₄·7H₂O	400 mg.	+ H₂O	54	"
MnSO₄·4H₂O	20 "	*l*(−)-Hydroxyproline	20	"
NaCl	20 "	*dl*-Isoleucine	50	"
FeSO₄·7H₂O	20 "	*dl*-Leucine	400	"
Adenine*	100 "	*dl*-Lysine hydrochloride	300	"
Guanine*	100 "	*dl*-Norleucine	120	"
Uracil*	100 "	*dl*-Phenylalanine	160	"
Thiamine chloride	2.0 mg.	*l*(−)-Proline	140	"
Pyridoxine hydrochloride	0.4 "	*dl*-Serine	240	"
Calcium pantothenate	0.4 "	*dl*-Threonine	180	"
Riboflavin	0.4 "	*l*(−)-Tryptophane	200	"
Nicotinic acid	0.8 "	*l*(−)-Cystine	400	"
p-Aminobenzoic acid	0.4 "	*l*(−)-Tyrosine	130	"
Biotin	0.01 "	*dl*-Valine	240	"
Folic acid†	30 γ‡	*l*(−)-Methionine§	100	"
Solution brought to 1000 cc. volume, pH 6.8				

* Baumgarten *et al.* (7).

† Obtained through the courtesy of Dr. R. J. Williams, The University of Texas.

‡ 30 γ of material of "potency 5000."

§ Omitted when determining methionine.

Bacto yeast extract, and 1.5 gm. of agar, per 100 cc. The cultures were stored in a refrigerator and subcultured each month. Inoculum for the assay was prepared by transferring a small amount of the growth from a stab culture to a centrifuge tube containing 10 cc. of a medium consisting of 1 gm. of glucose, 1 gm. of Bacto yeast extract, and 100 cc. of liver infusion, prepared as recommended by the Difco Laboratories. After incubation at 35° for 24 hours, the cells were centrifuged, washed with sterile salt solution, and suspended in 5 cc. of sodium chloride solution. 1 drop of the cell suspension was used to inoculate each tube in the assay.

Preparation of Samples for Assay—An amount of material equivalent to 1 gm. of protein was refluxed for 24 hours with 25 cc. of 20 per cent hydrochloric acid. The hydrolysate was concentrated to a small volume in an air bath, and the residue was dissolved in water and boiled for a few minutes with a small quantity of norit. The hot mixture was filtered by suction, and the filtrate made up to a volume of 100 cc. with distilled water. For assays, 10 cc. aliquots of this solution were adjusted to pH 6.8 and diluted to 1 liter. 1 cc. of the solution contained the protein equivalent of 100 γ per cc. The solutions were stored under toluene in a refrigerator.

Preparation of Methionine Standards—The methionine standard solutions were prepared by dissolving 10 mg. of $l(-)$-methionine in 1 liter of water. A 200 cc. aliquot of this solution was made up to 1 liter with distilled water. The two solutions containing 10 γ and 2 γ per cc., respectively, were used for preparing the standard curve.
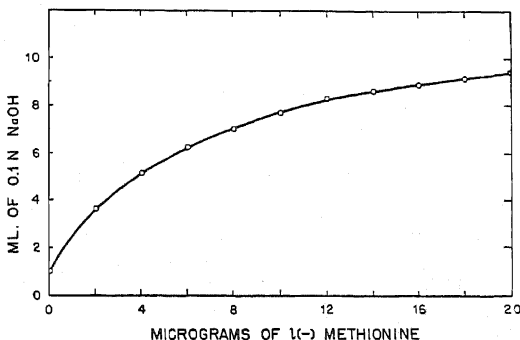
FIG. 1. Methionine curve

Assay Procedure—For the assay of methionine, 5 cc. quantities of the basal medium were used. A standard curve was prepared by adding appropriate amounts of the two standard solutions of methionine to separate tubes, in concentrations ranging from 0 to 20 γ. Each concentration was prepared in quadruplicate. To another set of tubes containing 5 cc. of the medium were added, 1.0, 2.0, 3.0, and 4.0 cc. quantities of the protein hydrolysate. The volume in all the tubes was then brought to 10 cc. with water. The tubes were autoclaved at 15 pounds pressure for 15 minutes and were inoculated with 1 drop of the *Lactobacillus arabinosus* suspension and incubated at 35–36° for 72 hours. The lactic acid was titrated with 0.1 N NaOH, with bromothymol blue as indicator. The cc. of 0.1 N NaOH required to neutralize the acid in the tubes of the standard solution were plotted against micrograms of methionine (Fig. 1).

Table II shows the values found for several proteins and foods when determined at different assay levels. The reproducibility of the amount

of methionine found in several proteins when determined by separate assays on each protein is given in Table III.   Recovery experiments were also

TABLE II

Methionine Content of Some Proteins and Foods Determined at Different Assay Levels*

Protein assay level	Casein		Zein		Ovalbumin		Ox muscle	
					Methionine found			
γ	γ	per cent	γ	per cent	γ	per cent	γ	per cent
100	2.40	2.40	1.40	1.40	4.40	4.40	2.50	2.50
200	4.70	2.35	2.60	1.30	8.50	4.25	5.10	2.55
300	7.35	2.45	3.90	1.30	12.60	4.20	7.60	2.53
400	10.00	2.50	5.60	1.40	16.40	4.10	10.0	2.50
Average.......		2.42		1.35		4.24		2.52
	Whole wheat		Whole yellow corn		White rice		Black-eyed peas	
500	0.80	0.16	0.80	0.16			1.10	0.22
1000	1.80	0.18	1.80	0.18	0.95	0.09	2.20	0.22
1500	2.50	0.17	2.50	0.17			3.30	0.22
2000	3.30	0.16	3.50	0.17	2.00	0.10	4.40	0.22
3000					2.90	0.09		
4000					3.92	0.10		
Average.......		0.17		0.17		0.10		0.22

* Percentages uncorrected for moisture and ash.

TABLE III

Reproducibility of Methionine Content of Proteins When Determined by Separate Assays*

Protein	Assay 1	Assay 2	Assay 3	Average
	per cent	per cent	per cent	per cent
Casein.........................	2.42	2.39	2.32	2.38
Ovalbumin....................	4.24	4.16	4.10	4.17
Lactalbumin..................	1.52	1.50	1.53	1.52
Zein..........................	1.35	1.26	1.40	1.34

* Uncorrected for moisture and ash.

made of methionine added in different proportions to hydrolysates of casein and gelatin (Table IV).   The results of the several experiments indicate a high degree of reliability of the method.

The methionine values (Table V) found for the proteins and foods assayed

are in close agreement with those obtained for samples of the same material by the colorimetric method described in the preceding paper (8), but they

TABLE IV

Recovery of Methionine Added to Protein Hydrolysates

Protein hydrolysate	Methionine				
	In hydrolysate	Added	Total	Found	Recovery
	γ	γ	γ	γ	*per cent*
Casein	2.40	2.00	4.40	4.40	100
		4.00	6.40	6.50	102
		6.00	8.40	8.50	101
		8.00	10.40	10.50	101
Gelatin	0.66	2.00	2.66	2.55	96
		4.00	4.66	4.60	98
		6.00	6.66	6.40	96
		8.00	8.66	8.30	96

TABLE V

Methionine Content* of Some Proteins and Foods (Percentages, Corrected for Moisture and Ash)

Material	N	Microbiological assay	Chemical assay	Material	N	Microbiological assay	Chemical assay
Arachin	18.30	0.24	0.31	Barley, pearled	1.86	0.12	0.10
Casein	16.07	2.58	2.60	Brazil nut meal	9.03	2.78	2.72
Conarachin	18.20	2.13	2.24	Corn, whole yellow	2.22	0.20	0.21
Cottonseed globulin	18.00	1.40	1.45	" germ, defatted	3.93	0.35	0.36
Coconut globulin	17.42	2.02	2.12	Cottonseed flour	10.36	0.85	0.88
Edestin	18.55	2.12	2.14	Egg, whole, dried	8.11	1.39	1.55
Ovalbumin (crystalline)	15.98	4.43	4.48	Milk, dry, skim	6.57	0.89	0.89
Gelatin (Bacto)	18.32	0.78		Oatmeal	2.73	0.18	0.20
Glycinin	17.30	1.15	1.20	Peanut flour	10.15	0.52	0.58
Lactalbumin	15.39	1.62	1.71	Peas, black-eyed	4.15	0.25	0.26
Peanut, total globulins	18.01	1.00	1.11	Rice, white	1.26	0.11	0.09
Phaseolin (navy bean)	16.07	1.12	1.21	Rye, whole	1.98	0.14	0.13
Ox muscle	16.00	2.75	2.75	Wheat, whole	3.07	0.19	0.23
Wheat bran globulin	17.76	1.04	1.21	" germ, defatted	6.50	0.51	0.56
Zein	16.00	1.41	1.62	Yeast, dried, brewers'	7.71	0.53	0.54

* In order to avoid repetition, methionine values previously recorded for the materials listed are omitted in this table, since they are given in the preceding paper.

are lower than those previously recorded in the literature. Most of the latter were determined by chemical methods. Recently, Stokes and co-

workers (6) have determined methionine in several proteins by a microbiological method in which *Streptococcus faecalis* is employed. The values here reported agree closely with those they found for the same proteins.

SUMMARY

A microbiological method is described for the determination of methionine in proteins with *Lactobacillus arabinosus*. The procedure has been applied to the assay of thirty proteins and foods with results that agree closely with those obtained for the same proteins by a colorimetric method.

BIBLIOGRAPHY

1. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
2. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
3. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
4. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511 (1943).
5. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944). Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 715 (1944).
6. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
7. Baumgarten, W., Garey, J. C., Olsen, M. J., Stone, L., and Boruff, C. S., *J. Am. Chem. Soc.*, **66**, 1607 (1944).
8. Horn, M. J., Jones, D. B., and Blum, A., *J. Biol. Chem.*, **166**, 313 (1946).

THE AMINO ACIDS OF HUMAN SWEAT

By STANLEY W. HIER, THEODORE CORNBLEET, AND OLAF BERGEIM

(*From the Research Laboratories, The Wilson Laboratories, and the Departments of Dermatology and Biological Chemistry, College of Medicine, University of Illinois, Chicago*)

We have previously described microbiological procedures for the determination of amino acids in blood plasma (1, 2). These methods are now applied to sweat and certain experiments are carried out with the view of investigating amino acid secretion by the sweat glands. The amounts of ten individual amino acids in sweat are reported for the first time.

EXPERIMENTAL

Methods

The microbiological methods used were those described previously by Hier and Bergeim (1, 2) and by Hier *et al.* (3), except that threonine was determined with *Streptococcus faecalis* according to Stokes *et al.* (4), since more consistent results were obtained with this organism than with *Lactobacillus arabinosus* as previously used. However, we have found no difference in values when *Streptococcus faecalis* or *Lactobacillus delbrückii* was substituted for *Lactobacillus casei* (used for arginine, phenylalanine, and tyrosine), *Lactobacillus arabinosus* (used for leucine, isoleucine, valine, and tryptophane), or *Leuconostoc mesenteroides* (used for lysine and histidine).

The subjects used in all experiments were male medical and dental students ranging from 20 to 30 years of age. Unless otherwise stated in Tables I to VI, all subjects had a shower or bath no longer than 2 hours previous to the collection of the sweat sample. This was a precaution against variable accumulation of amino acids on the skin by evaporation. Specimens of sweat were collected by encasing the nude subjects in a rubber bag as far as their necks and seating them in a heat cabinet. Incandescent lamps furnished sufficient heat to obtain 100 to 200 cc. of sweat in 20 to 30 minutes. The collected sweat was then filtered through a medium porosity Berkefeld filter to remove dirt and débris. 10 cc. of this filtrate were diluted to 50 cc. for assay. 1 cc. of basal medium diluted to a final volume of 2 cc. with the sample and water was used in all cases.

Normal Values—In Tables I and II, data are shown for normal subjects with slight variations in preparation of subjects, volume of collection, and after ingestion of tyrosine and histidine. In this series the first and second sweat samples for each subject were collected on different days. There

does not appear to be any effect of diet upon the amounts of amino acids in the sweat since there is no consistent trend shown in Subjects 1, 2, 3, 4, or 5, depending upon whether samples were collected before or after eating. Furthermore, the ingestion of 20 gm. of $l(-)$-tyrosine caused only a slight increase in the tyrosine content of sweat from Subject 7, which is well within the normal range. Similarly the ingestion of 20 gm. of $l(-)$-histidine hydrochloride in Subjects 8 and 9 caused no increase in the histidine content of the sweat.

TABLE I

Free Amino Acid Composition of Sweat from Normal Human Subjects Collected after Slightly Varying Preparation of Subjects and Volume of Collection

The amino acid values are reported in micrograms per cc.

Amino acid	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5
	A.m.; no break-fast	P.m.; after lunch	P.m.; after lunch	P.m.; no lunch	P.m.; after lunch	P.m.; no lunch	P.m.; after lunch	P.m.; after lunch	P.m.; after lunch
Arginine..............	170.0	171.0	92.5	60.5	139.0	159.0	170.0	140.0	110.0
Histidine.............	140.0	69.0	46.5	42.5	92.5	74.5	100.0	120.0	97.5
Isoleucine............	37.3	22.0	21.3	16.3	16.5	25.5	25.8	27.0	31.0
Leucine..............	37.5	33.3	20.8	21.3	19.8	25.9	34.0	32.8	30.5
Lysine...............	33.8	25.1	21.1	20.8	22.7	27.0	19.8	20.0	19.6
Phenylalanine.........	34.7	21.5	17.5	17.0	19.3	18.8	24.5	21.8	33.3
Threonine............	71.8	65.7	50.0	21.3	70.0	81.8	50.0	31.5	40.5
Tryptophane..........	16.0	12.0	7.5	8.5	10.0	13.0	13.0	15.0	18.5
Tyrosine.............	25.0	32.5	13.2	18.0	27.5	32.0	33.5	39.5	54.5
Valine...............	43.5	31.0	24.0	24.0	26.3	37.3	32.8	35.5	38.3
Total	609.6	483.1	314.4	250.2	443.6	494.8	503.4	483.1	473.7
Volume, *cc*...........	75.0	54.0	80.0	70.0	185.0	160.0	92.0	160.0	25.0
NaCl, %..............	0.25	0.22	0.44	0.43	0.37	0.39	0.82	0.90	

Subject 6 demonstrates that there is considerable accumulation of amino acids on the skin due to evaporation. This subject did not bathe for 2 days previous to the first collection and all the amino acids are markedly higher than the normal for all subjects, as well as higher than was obtained with the same subject in a later collection after a shower in the usual manner.

From the data in Tables I and II it may also be seen that there is neither negative nor positive correlation between the volume of sweat and the amount of amino acids present. This, of course, may not be true if larger volumes of sweat are collected. In our data the maximum variation in volume is from 25 to 240 cc. of sweat.

Variation in Single Subject—The data in Table III demonstrate the variation in amounts of amino acids in sweat which occurs in the same subject

<div align="center">

TABLE II

Free Amino Acid Composition of Sweat from Normal Human Subjects Collected after
Slightly Varying Preparation of Subjects, Volume of Collection, and after
Large Doses of Tyrosine and Histidine

</div>

The amino acid values are reported in micrograms per cc.

Amino acid	Subject 6		Subject 7		Subject 8		Subject 9	
	P.m.; 2 days, no shower	P.m.; after shower	P.m.; after lunch	P.m.; after 20 gm. tyrosine	P.m.; after lunch	P.m. after 20 gm. histidine HCl	P.m.; after lunch	P.m.; after 20 gm. histidine HCl
Arginine	315.0	141.0	130.5	191.0	135.0	205.0	112.0	149.5
Histidine	210.0	46.5	62.5	66.0	100.0	128.0	34.5	41.5
Isoleucine	52.0	22.5	16.0	20.4	26.3	22.0	20.0	33.3
Leucine	57.3	21.0	30.3	29.5	22.0	33.3	33.3	35.8
Lysine	45.5	17.0	26.8	20.5	24.0	24.3	24.5	27.5
Phenylalanine	38.0	13.0	15.5	25.5	19.3	30.0	25.3	27.3
Threonine	155.0	55.3	45.5	77.3	58.0	75.0	41.7	73.0
Tryptophane	27.5	9.0	9.8	11.0	10.5	13.5	8.5	13.5
Tyrosine	76.0	27.5	24.0	44.5	26.0	40.0	30.5	42.5
Valine	63.3	23.0	30.3	33.3	34.3	34.3	26.8	28.0
Total	1039.6	375.8	391.2	519.0	455.4	605.4	357.1	471.9
Volume, cc	85.0	95.0	240.0	80.0	125.0	75.0	110.0	124.0
NaCl, %	0.71	0.52	0.39	0.38	0.84	0.34	0.58	0.34

<div align="center">

TABLE III

Variation in Amino Acid Composition of Sweat Obtained from Same Subject (No. 10)
over Period of 3 Months (Volumes 80 to 150 Cc.)

</div>

The amino acid values are supported in micrograms per cc.

Date of collection	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine	Total
1946											
Jan. 15	170.0	89.0	25.9	27.5	19.2	20.0	64.0	16.0	17.0	31.0	479.6
" 22	126.0	86.0	23.4	25.0	21.2	17.5	59.0	16.0	21.0	26.0	421.1
" 28	99.5	85.0	14.3	14.8	15.0	21.3	54.0	25.5	28.5	22.5	380.4
Feb. 5	190.0	74.5	13.4	20.0	16.5	18.8	51.3	17.5	29.5	22.8	455.2
" 14*	76.5	52.5	13.3	9.8	12.3	12.0	29.0	9.0	25.0	30.3	269.7
" 14†	105.0	55.5	22.0	14.5	13.3	17.7	37.5	10.5	26.5	31.5	334.0
Apr. 7‡	119.0	63.5	14.3	16.0	17.3	18.0	52.5	9.5	34.0	23.3	367.4

  * Before lunch.
  † 2 hours after lunch.
  ‡ 2 hours after 25 gm. of histidine hydrochloride.

when a series of collections is made over a prolonged period. Seven
collections were made within a period of 3 months. In Table IV the means

and standard errors calculated from the data in Table III for Subject 10 are compared with the normal values. These normal values include all of the subjects in Tables I and II except the first sample for Subject 6. They also include the sweat values in Table V. In the case of arginine, isoleucine, leucine, and lysine, the variation is of the same order of magnitude for the single subject as compared to all the subjects, while there is greater variation for the single subject in the case of tryptophane and less variation for histidine, phenylalanine, threonine, tyrosine, and valine.

Table III also shows that diet or previous ingestion of 20 gm. of $l(-)$-histidine hydrochloride has no effect on amino acid composition of sweat in this subject.

TABLE IV

Range of Free Amino Acids in Sweat from Nine Normal Subjects Compared to That of Seven Sweat Collections from One Subject and to Normal Human Plasma

The mean and standard deviation are given in micrograms per cc.

Amino acid	Subject 10; data from Table III	Normal subjects; Tables I and II	Normal human plasma (cf. (1))
Arginine	126.6 ± 37.0	135.8 ± 39.2	23.2 ± 6.4
Histidine	72.3 ± 4.4	80.2 ± 9.7	14.2 ± 2.4
Isoleucine	17.5 ± 5.0	22.7 ± 6.6	16.6 ± 3.4
Leucine	18.2 ± 5.9	26.9 ± 7.7	20.3 ± 3.6
Lysine	16.4 ± 2.9	22.6 ± 4.5	29.7 ± 4.6
Phenylalanine	17.9 ± 2.7	21.9 ± 6.3	14.0 ± 3.5
Threonine	49.6 ± 11.4	53.8 ± 18.4	20.8 ± 4.9
Tryptophane	14.9 ± 5.3	11.2 ± 3.3	11.1 ± 2.3
Tyrosine	25.9 ± 5.4	31.5 ± 9.5	15.0 ± 4.2
Valine	26.8 ± 3.6	29.6 ± 7.5	28.9 ± 3.7

Amino Acids of Sweat Compared to Plasma—It may also be seen from Table IV that the mean values for isoleucine, leucine, lysine, phenylalanine, tryptophane, and valine are of the same order of magnitude in sweat and plasma. However, arginine and histidine are approximately 6 times higher in sweat than in plasma, while threonine and tyrosine are about 2 times higher in sweat.

To study this problem further one of us (S. W. H.) ingested 25 gm. of $l(-)$-tyrosine and $l(-)$-histidine hydrochloride after having simultaneous blood, sweat, and urine samples taken. 2 hours later sweat, urine, and blood samples were again taken. It is evident from Table V that again the arginine and histidine content of the sweat is markedly higher than that of the blood plasma, while the threonine and tyrosine content of sweat is slightly higher than that of plasma. Furthermore, although the level of histidine and tyrosine rose significantly in the blood and spilled over into the urine as a result of the large dose of ingested amino acids, there was no such change in the sweat.

TABLE V

Effect of Ingestion of Large Amounts of l(−)-Histidine Hydrochloride (25 Gm.) and l(−)-Tyrosine (25 Gm.) on Free Amino Acid Composition of Sweat, Blood Plasma, and Urine

The amino acid values are reported in micrograms per cc.

Sample	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine	Total
Sweat 1; before ingestion of amino acids	92.0	100.0	14.3	12.5	18.2	16.3	35.0	6.0	28.0	17.0	339.3
Sweat 2; after ingestion of amino acids	77.0	82.5	10.8	11.3	14.4	14.0	25.8	6.5	27.5	13.5	283.3
Plasma 1; before ingestion of amino acids	31.8	19.5	8.0	21.5	26.7	13.2	18.9	12.9	17.4	25.5	
Plasma 2; after ingestion of amino acids	42.0	555.0	10.0	16.7	34.2	11.4	27.0	10.8	55.2	25.5	
Urine 1; before ingestion of amino acids		220.0							41.5		
Urine 2; during ingestion of amino acids		160.0							35.5		
Urine 3; after ingestion of amino acids		1750.0							176.5		

TABLE VI

Effect of Ingestion of Large Amounts of l(−)-Tyrosine (25 Gm.), dl-Isoleucine (25 Gm.), and dl-Threonine (25 Gm.) on Amino Acid Composition of Sweat and Blood Plasma

The amino acid values are reported in micrograms per cc.

Sample	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine	Total
Sweat 1; before ingestion of amino acids	104.0	59.5	17.3	19.5	11.3	19.5	35.0	9.0	23.0	15.3	313.4
Sweat 2; after ingestion of amino acids	112.0	67.0	22.5	27.8	13.8	22.5	38.3	9.5	27.0	17.8	358.2
Plasma 1; before ingestion of amino acids	26.4	14.4	15.3	20.3	22.5	21.3	17.3	5.7	15.0	25.4	
Plasma 2; after ingestion of amino acids	21.0	14.7	171.0	7.7	15.9	22.8	99.0	4.2	76.5	15.5	

The influence of 25 gm. of l(−)-tyrosine, 25 gm. of dl-isoleucine, and 25 gm. of dl-threonine was similarly studied in a second experiment on the same subject, as shown in Table VI. Again, while the blood level of these

amino acids rose significantly, there was no effect on the composition of the sweat. Here also the arginine, histidine, threonine, and tyrosine concentrations are higher in the sweat than in the plasma, while the other amino acids are present in about the same concentration in plasma and sweat.

DISCUSSION

Our results show that the amino acids secreted in the sweat are independent of the effect of diet or food ingestion since no difference in composition is found in sweat taken from subjects in a fasting state or after eating. Furthermore, the level of the amino acids in sweat is independent of the variations in the blood. Ingestion of large amounts of amino acids which causes a marked rise in the blood level is without effect on the sweat amino acids. This must mean that the amino acids do not appear in the sweat merely by filtration from the blood. This is further shown by the fact that arginine and histidine are markedly higher and threonine and tyrosine are slightly higher in sweat than in plasma, while the other amino acids studied are present in approximately the same order of magnitude in both fluids. The high arginine content of sweat is particularly interesting in view of the findings of Talbert et al. (5) that the urea nitrogen of sweat is about 4 or 5 times that of blood. Arginine may be involved in the formation of this urea by the sweat gland.

These results are consistent with the findings of other observers that there is not a very good correlation between blood and sweat levels of other substances. The glucose (6) content of sweat is considerably lower than that of the blood, while lactic acid (7) is about 20 times higher in sweat than in blood. According to Talbert (8), using a rather unreliable method, the amino nitrogen of sweat is slightly lower than that of blood. Alcohol when ingested appears in the blood and sweat in the same concentrations (9). Johnson et al. (10) recently have shown that the sweat chloride concentration is lower than that of serum, and that there is no apparent correlation between the level of serum and sweat chlorides.

The variation in the amino acid composition of sweat samples taken from one individual over a period of time appears to be approximately equivalent to the variation occurring within the normal population for arginine, isoleucine, leucine, and lysine. Less variation occurs with histidine, phenylalanine, threonine, tyrosine, and valine in the individual than in the population. In our subject the tryptophane content of the sweat was more variable than that of the population.

The amount of amino acids lost in the sweat is not strikingly significant in the economy of amino acids by the body. In our subjects the sweat contained about 0.44 mg. per cc. of the ten amino acids studied. Under average conditions of temperature and humidity, the amount of sweat is about

500 cc. per day.   This would represent a loss of about 220 mg. per day of the ten amino acids.   Even with excessive sweating in warm weather with active work when 3 liters of sweat may be formed, only 1.3 gm. of these amino acids would be lost.

SUMMARY

The microbiological determination of ten free amino acids in normal sweat is reported.   These are found as follows in micrograms per cc.: arginine 135.8 ± 39.2, histidine 80.2 ± 9.7, isoleucine 22.7 ± 6.6, leucine 26.9 ± 7.7, lysine 22.6 ± 4.5, phenylalanine 21.9 ± 6.3, threonine 53.8 ± 18.4, tryptophane 11.2 ± 3.3, tyrosine 31.5 ± 9.5, and valine 29.6 ± 7.5. It is found that the marked increases in the free amino acid content of the plasma due to ingestion of large amounts of single amino acids are without effect on the amino acid content of the sweat.   Similarly, food ingestion does not appear to have any effect.   Furthermore, the arginine and histidine content particularly, and the threonine and tyrosine content of sweat to a lesser extent, is higher in sweat than in blood plasma, while the other amino acids are present in about the same amounts.   These observations indicate that the amino acids do not appear in sweat merely as a result of filtration from the blood plasma.   The loss of amino acids in the sweat does not appear to be of much importance in the economy of amino acids by the body.

BIBLIOGRAPHY

1. Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, **161**, 717 (1945).
2. Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, **163**, 129 (1946).
3. Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, **161**, 705 (1945).
4. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
5. Talbert, G. A., Finkle, J. R., and Katsuki, S. S., *Am. J. Physiol.*, **82**, 153 (1927).
6. Silvers, S., Forster, W., and Talbert, G. A., *Am. J. Physiol.*, **84**, 577 (1928).
7. Fishberg, E. H., and Bierman, W., *J. Biol. Chem.*, **97**, 433 (1932).
8. Talbert, G. A., and Haugen, C., *Am. J. Physiol.*, **81**, 510 (1927).
9. Nyman, E., and Palmlov, A., *Skand. Arch. Physiol.*, **74**, 155 (1936).
10. Johnson, R. E., Pitts, G. C., and Consolazio, F. C., *Am. J. Physiol.*, **141**, 575 (1944).

OLEIC ACID AS A GROWTH STIMULANT FOR LACTOBACILLUS CASEI

By VIRGINIA R. WILLIAMS AND E. A. FIEGER

(From the Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge)

Lipoidal stimulation of *Lactobacillus casei* in the microbiological biotin assay was noted at this laboratory, and methods for removing stimulating substances reported (1). Because of difficulties encountered from time to time in reproducing the effects observed with pure oleic acid, experiments were devised to determine what factors affect the extent of acid production stimulated by oleic acid. Accordingly, a study was made of the influence of (a) pH of the medium, (b) length of incubation period, (c) temperature of incubation, and (d) concentration of lipide on the growth of *Lactobacillus casei* in the presence of oleic acid as measured acidimetrically and in some cases turbidimetrically. Those findings are reported here in addition to several related experiments. Parallel studies made with rice oil will be presented in a later communication.

EXPERIMENTAL

General Procedure—Unless altered specifically, the standard procedure for each experiment was as follows: Tubes containing increments of standard biotin from 0 to 1000 micromicrograms and tubes containing 100 γ of oleic acid plus either 0 or 1000 micromicrograms of pure biotin were supplied with biotin-free medium of pH exactly 5.6, autoclaved 15 minutes at 15 pounds of pressure, cooled rapidly to room temperature, inoculated with a washed, saline suspension of *Lactobacillus casei* as described by Landy and Dicken (2), and incubated at 37° for 72 hours. At the end of this period the acid produced by the bacteria was titrated with 0.1 N NaOH by use of a Beckman pH meter. Turbidimetric measurements were made against distilled water at 7000 A on the Coleman model 11 spectrophotometer. All assays were made in duplicate.

For the sake of conciseness, standard tubes will be referred to here as Tubes S, with the micromicrograms of biotin given as a subscript, *e.g.* Tubes S_{1000}. Tubes containing 100 γ of oleic acid will be referred to as Tubes OA, with micromicrograms of included biotin indicated in subscripts, *e.g.* Tubes OA_0 or OA_{1000}.

A basal medium was used containing proportions of vitamins recommended by Wright and Skeggs (3) and proportions of other constituents

as recommended by Krehl, Strong, and Elvehjem (4). Additional constituents were 20 mg. of xanthine, 400 mg. of asparagine, and 50 γ of folvite[1] per liter of double strength basal medium. Standard curves obtained with this medium were nearly linear, with a range of 2.93 to 16.79 ml. of 0.1 N acid, taken as an average of thirty-one typical curves.

The oleic acid, chemically pure and linoleic acid-free, was obtained from Eimer and Amend. Except for short periods during weighing, it was kept refrigerated at 0°. The iodine value was 89.8; calculated 89.9. To prepare an emulsion for pipetting into assay tubes, 100 mg. of oleic acid were weighed out and dissolved in 10 ml. of 95 per cent alcohol. 1 ml. of this solution was pipetted into 99 ml. of distilled water, giving an opalescent solution containing 100 γ of oleic acid per ml.

Effect of pH—The pH of the medium was varied from 4.5 to 7.0 at intervals of 0.5 unit. In Fig. 1 are presented curves taken as an average of three experiments. No growth was evident in any of the tubes at pH 4.5, doubtless because of the acidity of the medium. At pH 5.0 some growth was obtained as evidenced by the increase in acid in Tubes S_{1000} over Tubes S_0. The high acidity of Tubes S_0 was due obviously to the low pH of the medium. Growth in Tubes OA_{1000} did not exceed that of Tubes S_0 and there was likewise no stimulation in Tubes OA_0. At pH 5.5, normal values were obtained for the standard tubes and the effect of the oleic acid was most pronounced. Great stimulation was obtained in Tubes OA_0. Inhibition was observed in Tubes OA_{1000}, although growth at this pH was maximum for the curve of Tubes OA_{1000}. As will be discussed later, the inhibition evidenced in the latter tubes was overcome with a longer incubation period. Repetition of these curves at intervals of 0.1 pH unit showed the same general shape, with maxima for oleic acid around pH 5.5. When the pH was raised to 6.5 or 7.0, the growth of the organism in the presence of oleic acid was depressed noticeably below that in Tubes S_0.

Effect of Length of Incubation Period—Five replicate sets of tubes were inoculated and incubated, and one set titrated at each 24 hour period (Table I). Acid production in Tubes OA_0 did not exceed that in Tubes S_0 until the 72 hour reading was made, whereas acid production in Tubes OA_{1000} was not equal to or in excess of that in Tubes S_{1000} until the 96 hour reading. The reason for the increased lag in the latter case is unknown.

Effect of Temperature of Incubation—Replicate sets of tubes were incubated at temperatures ranging from 30–45°. The maximum stimulating effect with oleic acid was observed at a temperature of 37°, whereas no

---

[1] Synthetic *Lactobacillus casei* factor kindly supplied by the Lederle Laboratories, Inc.
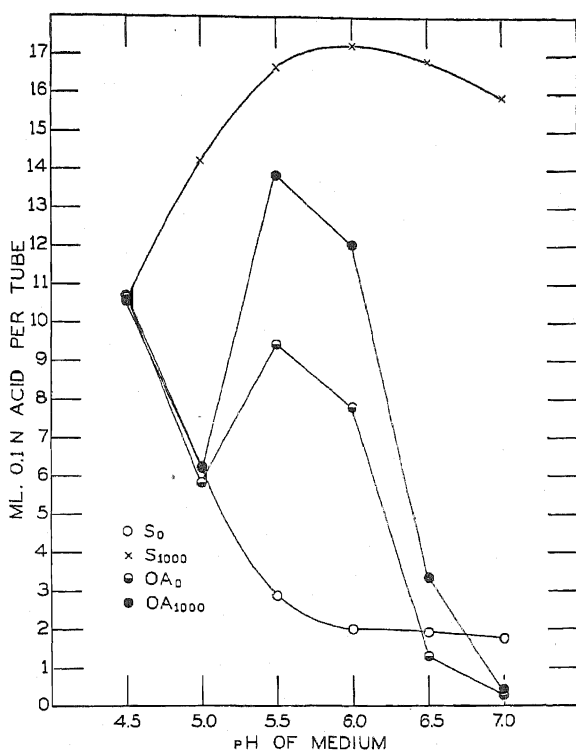
FIG. 1. Effect of pH on the acidimetric response of *Lactobacillus casei* to biotin-free basal medium (Tubes S_0), biotin-free basal medium containing oleic acid (Tubes OA_0), complete basal medium (Tubes S_{1000}), and complete basal medium containing oleic acid (Tubes OA_{1000}).

TABLE I

Effect of Incubation Time on Oleic Acid Stimulation of Lactobacillus casei

Time	Tubes S_0	Tubes OA_0		Tubes S_{1000}	Tubes OA_{1000}	
	0.1 N acid per tube	0.1 N acid per tube	Apparent biotin content, per tube	0.1 N acid per tube	0.1 N acid per tube	Apparent biotin content, per tube
hrs.	*ml.*	*ml.*	*micromicrograms*	*ml.*	*ml.*	*micromicrograms*
24	1.59	1.25	<0	4.17	1.15	<0
48	2.25	1.64	<0	13.85	1.53	<0
72	2.70	8.63	243	17.45	15.05	635
96	3.09	13.15	418	19.20	19.56	1040*
120	4.02	16.42	658	20.07	21.73	1170*

* Extrapolated value.

growth could be obtained in the presence of oleic acid at 45° (Table II). In all cases the pH of the medium was favorable to stimulation (5.6).

Effect of Concentration of Lipide—The concentration of oleic acid was varied between 10 and 5000 γ per tube (Table III). Maximum acid production per 10 ml. of medium was obtained at a concentration of 400 γ,

TABLE II

Effect of Incubation Temperature on Oleic Acid Stimulation of Lactobacillus casei

Temperature of incubation	Tubes S_0	Tubes OA_0		Tubes S_{1000}	Tubes OA_{1000}	
	0.1 N acid per tube	0.1 N acid per tube	Apparent biotin content, per tube	0.1 N acid per tube	0.1 N acid per tube	Apparent biotin content, per tube
°C.	ml.	ml.	micromicrograms	ml.	ml.	micromicrograms
30–31	2.30	2.26	0	10.99	2.26	0
33	1.43	3.98	137	13.72	6.00	253
37	2.55	8.36	307	16.88	13.58	657
45	2.91	1.69	<0	15.23	1.80	<0

TABLE III

Effect of Concentration on Oleic Acid Stimulation of Lactobacillus casei

Oleic acid per tube	Apparent biotin content per tube
γ	micromicrograms
10	50
50	236
100	349
200	481
300	497
400	585
500	536
1000	440
2000	275
3000	87
4000	10
5000	<0

with marked inhibition evidenced at the higher concentrations. The data presented represent averages of three determinations.

Comparison of Titrimetric and Turbidimetric Assays in Presence of Oleic Acid—Previous comparisons of the titrimetric and turbidimetric methods of biotin assay with *Lactobacillus casei* at this laboratory had given excellent agreement on materials such as Curbay powder or fat-free rice polish. Data taken from several such assays are presented as Assays 7, 8, and 9 in Table IV. The acidimetric method, however, has in the past been used almost exclusively here. In the course of the studies on oleic

acid it was observed many times that oleic acid-containing samples indi-
cating heavy growth by their turbidity contained surprisingly small
amounts of acid. A comparison then was made of the two methods on
tubes containing oleic acid only (Tubes OA_0) or oleic acid plus added
biotin (Tubes OA_{1000}). In all cases much higher assay values were ob-
tained turbidimetrically, and this discrepancy persisted even when the
tubes had been incubated 7 days. These data are presented in Table IV.

TABLE IV

*Comparison of Turbidimetric-Titrimetric Assay Methods with Tubes Containing
Oleic Acid*

Assay No.	Tube contents	Apparent biotin content per tube as measured by	
		Titration	Turbidity
		micromicrograms	*micromicrograms*
1	OA_0, 72 hrs.	333	836
		442	905
		238	902
2	OA_{1000}, 72 hrs.	640	1460*
		678	2550*
		630	1460*
3	OA_0, 96 hrs.	636	1217*
		595	1170*
4	OA_{1000}, 96 hrs.	1295*	1700*
		1460*	1700*
5	OA_0, 168 hrs.	920	1204*
		1040*	1080*
6	OA_{1000}, 168 hrs.	1280*	1580*
		1260*	1450*
7†	C_1, 72 hrs.	230	215
8†	C_2, 72 "	455	445
9†	C_3, 72 "	625	625

* Extrapolated value.
† Curbay powder extract.

Cell volume measurements made by centrifugation of cultures in Bauer-
Schenck protein tubes checked with turbidimetric assay.

In view of the extensive growth of cells and correspondingly low pro-
duction of acid in the 72 hour oleic acid cultures, it was postulated that the
organisms might be converting glucose to some product other than lactic
acid, at least during the first 3 days of growth. Glucose determinations
(Shaffer-Hartmann method) made in parallel with titrations over an 120
hour period at 24 hour intervals definitely showed that such was not the
case. In so far as glucose was concerned, no departure from normal
metabolism of the organisms could be detected.

Maintenance of Lactobacillus casei on "Biotin-Free" Medium—In view of the excellent growth of *L. casei* obtained in the presence of oleic acid on "biotin-free" medium (traces of biotin in the casein hydrolysate), it was decided to attempt continued maintenance of a culture on such a medium. *L. casei* was inoculated into the biotin-free medium described earlier, to which were added 100 γ of oleic acid per 10 ml. of medium, and was transferred approximately every 4 days to a fresh tube of such medium. At the date of this writing, 4 months from the first transfer, the culture is growing even more vigorously than initially. As tested recently, the cultural characteristics of the organism were unchanged from those of the cultures carried on glucose-yeast extract agar. A new transfer of Snell's strain obtained from the American Type Culture Collection grew well on the oleic acid medium. In all cases, a 36 to 48 hour lag occurs before cell production is noticeable on the oleic acid medium.

Although lack of biotin synthesis in the presence of stimulating oils had been demonstrated in a previous communication (1), the experiments were repeated with pure oleic acid. Two 96 hour cultures containing (a) 1000 micromicrograms of biotin per 10 ml. of medium and (b) 100 γ of oleic acid but no biotin per 10 ml. of medium were each separated as to cells and supernatant medium and hydrolyzed in 5 N sulfuric acid for 2 hours at 15 pounds of pressure. Hydrolysates were neutralized with Ba-$(OH)_2$, digested, filtered, concentrated, and assayed. No biotin was found in either cells or supernatant liquid of the oleic acid-supported culture. Biotin recovery from the biotin-containing culture was 61.0 per cent. These findings are in complete agreement with the previous work cited.

Stimulation of *Lactobacillus casei* by lipoidal materials has been reported in the riboflavin and pantothenic acid assays by Bauernfeind *et al.* (5) (to mention only one of many such papers), but in all these cases it has been pointed out by the workers that no stimulation occurred in tubes containing no added pure vitamin; *i.e.*, small amounts of either riboflavin or pantothenic acid were necessary for the lipoidal stimulant to exercise its effect. In the biotin assay, however, it has always been possible to produce lipoidal stimulation in tubes containing only the biotin-free basal medium plus the stimulating agent (1). It was held advisable to check this point on the present medium. Table V bears out the previous findings, growth with oleic acid alone having been obtained only in the case of the biotin-free medium. In this particular experiment acid production with oleic acid was even higher than usual, as will be noted by comparison with Tables I and II. The somewhat higher blanks (Tubes S_0) obtained with the biotin-free medium compared with the other media in Table V demonstrate the presence of traces of biotin mentioned above in connection with casein hydrolysate. Whether these traces are sufficient to make the case for

biotin completely analogous to those for riboflavin and pantothenic acid is not known at present. Work has been initiated on the effects of oleic acid in a completely synthetic medium.

Oleic acid stimulation in medium containing chloroform-extracted casein hydrolysate and in chloroform-extracted medium has been investigated because of the report of Kodicek and Worden (6) of differences found with extracted and unextracted media. No differences were found

TABLE V

Effect of Oleic Acid in Various Vitamin-Free Media

Tube contents	0.1 N acid per tube			
	Biotin-free medium	Pantothenic acid-free medium	Riboflavin-free medium	Nicotinic acid-free medium
	ml.	*ml.*	*ml.*	*ml.*
S_0	3.62	3.06	2.40	3.06
OA_0	11.63	2.66	2.18	2.97

TABLE VI

Effect of Other Fatty Acids on Acid Production of Lactobacillus casei

Acid added, 3.5×10^{-7} mole per tube	Acid production in 72 hrs.	
	Tubes with 0 biotin	Tubes with 1000 micromicrograms biotin
Caprylic	Normal	Normal
Capric	"	"
Lauric	No acid produced	Greatly inhibited
Myristic	" " "	" "
Palmitic	Normal	Normal
Stearic	"	"
Linoleic	No acid produced	No acid produced
Elaidic	Stimulated	Stimulated
Oleic*	"	Inhibited
" †	"	"

* Casein hydrolysate of medium chloroform-extracted.
† Entire medium chloroform-extracted.

with the present medium, comparable stimulation from oleic acid being obtained with either the chloroform-extracted medium or medium containing chloroform-extracted casein hydrolysate. These experiments are indicated in Table VI.

Effect of Other Fatty Acids—In Table VI are reported findings with other fatty acids. Of the saturated fatty acids, lauric and myristic were found to inhibit acid production in 72 hour cultures. In no case was stimulation

noted, although the pH of the medium was 5.6. Lower concentrations of lauric and myristic acids permitted half maximum growth in tubes containing 1000 micromicrograms of biotin. Linoleic acid completely repressed growth. Elaidic acid, on the other hand, stimulated growth both in tubes containing no added biotin and in those containing 1000 micromicrograms. Stimulation in the latter case in a 72 hour culture exceeded that observed repeatedly with oleic acid, as discussed before; *i.e.*, inhibition of growth in the presence of 1000 micromicrograms of biotin until 96 hours of incubation.

DISCUSSION

How oleic acid functions in the biotin metabolism of *Lactobacillus casei* and why its influence is affected by pH of medium, length of time and temperature of incubation, and concentration used are not known at the present time. The effects obtained suggest the possibility that under specific conditions oleic acid or some derivative can replace biotin. Two objections, however, can be raised to such a hypothesis: first, until oleic acid stimulation can be demonstrated on a truly biotin-free medium, it is not judicious to speak of oleic acid as having "replaced" biotin; and second, such a hypothesis leaves no explanation for similar phenomena observed in the riboflavin and pantothenic acid assays.

The lipoprotein nature of the cell cytoplasmic membrane (7) in addition to the surface properties of oleic acid (8) suggests possible physicochemical mechanisms, as discussed by Kodicek and Worden (6). The fact that elaidic acid, which can penetrate and pack in surface films better than oleic acid (9), proved more stimulating to *Lactobacillus casei* lends weight to such interpretations. Of the saturated fatty acids tested, only lauric and myristic displayed any effect, although palmitic and stearic acids are known to be surface-active also. The explanation for this behavior probably lies in the differences in stability of the emulsions. Myristic, lauric, and linoleic acids all formed opalescent colloidal solutions showing no tendency to break down. Stearic and palmitic acids, on the other hand, were stable for less than an hour in the dispersed state and would frequently flocculate in the test-tubes soon after being pipetted.

The sensitivity of this phenomenon to pH, temperature, time, and concentration of oil suggests the involvement of an enzyme system. The curves obtained with oleic acid-containing media at various pH values (Fig. 1) are strongly reminiscent of enzyme pH-activity curves. Further theorizing at this point is inadvisable.

SUMMARY

Oleic acid stimulation of *Lactobacillus casei* has been found affected by the pH of the medium, length of incubation period, temperature of in-

cubation, and concentration of oleic acid added. When the control conditions were varied one at a time, maximum stimulation was obtained at pH 5.5, incubation period of 96 to 120 hours, incubation temperature of 37°, and concentration of 400 γ of oleic acid per tube.

Parallel titrimetric and turbidimetric measurements of tubes containing oleic acid showed complete lack of agreement, with much higher values obtained in turbidimetric assay.

Lactobacillus casei has been successfully maintained on an essentially biotin-free medium containing oleic acid for a 4 month period and is continuing to grow well. Transfers are made every 3 to 4 days.

No synthesis of biotin from oleic acid could be demonstrated.

Oleic acid stimulation in a medium free of one vitamin was obtained only in the case of biotin. No stimulated growth could be obtained with either riboflavin-free, pantothenic acid-free, or nicotinic acid-free medium.

Of a number of fatty acids tested, lauric, myristic, and linoleic were found strongly inhibiting to the growth of *Lactobacillus casei*, whereas elaidic acid was more stimulating than oleic acid.

BIBLIOGRAPHY

1. Williams, V. R., and Fieger, E. A., *Ind. and Eng. Chem., Anal. Ed.*, **17**, 127 (1945).
2. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
3. Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).
4. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 471 (1943).
5. Bauernfeind, J. G., Sotier, A. L., and Boruff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 666 (1942).
6. Kodicek, E., and Worden, A. N., *Biochem. J.*, **39**, 78 (1945).
7. Knaysi, G., *Bot. Rev.*, **4**, 83 (1938).
8. Rideal, E. K., *Chem. and Ind.*, **58**, 830 (1939).
9. Florence, R. T., and Harkins, W. D., *J. Chem. Phys.*, **6**, 856 (1938).

STEROIDS DERIVED FROM BILE ACIDS

V. INTRODUCTION OF OXYGEN AT C11

By RICHARD B. TURNER, VERNON R. MATTOX, LEWIS L. ENGEL, BERNARD F. McKENZIE, AND EDWARD C. KENDALL

(From the Division of Biochemistry, Mayo Foundation, Rochester, Minnesota)

Following the preparation of $3(\alpha)$-hydroxy-11,12-dibromocholanic acid (1) various attempts were made to utilize this substance directly for the synthesis of steroid derivatives with oxygen at C_{11}. Such attempts, however, proved unsuccessful. Treatment of the dibromide with alkali resulted in the formation of $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid (2, 3) and efforts in this laboratory and elsewhere[1] to effect a selective replacement of 1 atom of bromine by the use of silver and other salts led to the simultaneous elimination of both halogens.

We have recently observed (4) that bromination of methyl 3,9-epoxy-Δ^{11}-cholenate (I) (Fig. 1) proceeds readily at low temperature and that crystallization of the reaction mixture from chloroform-methanol affords a dibromo derivative (II) melting at 143° in a yield of about 65 per cent. Concentration of the mother liquor gave additional material from which a second dibromide (III), melting at 123°, was obtained by repeated crystallization. Both dibromides are quantitatively converted to methyl 3,9-epoxy-Δ^{11}-cholenate by the action of zinc dust and methanol, and it is assumed that they are stereoisomers. Since the elimination of hydrogen bromide between C_9 and C_{11}, which can occur in $3(\alpha)$-hydroxy-11,12-dibromocholanic acid, is not possible in these compounds, it was of interest to determine whether by a direct replacement or through the formation of an oxide either of the dibromides (II or III) could be converted to a compound with oxygen at C_{11}.

Preliminary investigation indicated that the bromine in the low melting derivative (III) was relatively stable and all attempts to replace 1 atom preferentially were unsuccessful. The higher melting dibromide (II), however, when treated with sodium acetate in acetic acid, gave an acetoxybromo compound (XIV) in excellent yield (4). Substitution of silver oxide and aqueous acetone for sodium acetate-acetic acid led to the formation of a bromohydrin (XV) which in turn yielded XIV with acetic anhydride and sulfuric acid and an α-bromoketone (XVI) on oxidation with chromic acid. The bromoketone was subsequently obtained directly in 94 per cent

---

[1] Fieser, L. F., and Turner, R. B., unpublished data.

yield by treatment of the dibromide (II) with silver chromate and chromic acid in aqueous acetone. This series of reactions provides a route to C_{11}-oxygenated steroids, for the bromoketone (XVI) has been converted to a number of C_{11}-keto and C_{11}-hydroxy derivatives.
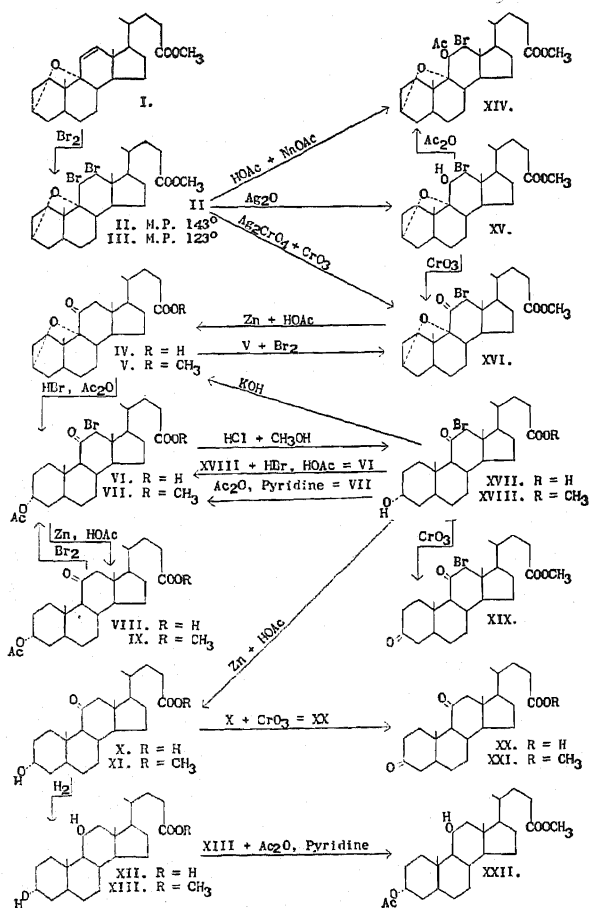
FIG. 1

With zinc dust and acetic acid XVI was easily debrominated and a compound (V) was obtained which must be either methyl 3,9-epoxy-11-ketocholanate or methyl 3,9-epoxy-12-ketocholanate. Conclusive evidence in favor of the C_{11}-keto structure was provided by cleavage of the epoxy ring.

Some difficulty was at first encountered in this step, for aluminum

bromide, zinc chloride, and acetyl chloride, hot aqueous mineral acids, and similar reagents either caused extensive decomposition or did not open the epoxide. The cyclic ether was unaffected by the Grignard reagent, and high pressure hydrogenation over Raney's nickel proceeded no farther than reduction of the keto group. The use of anhydrous hydrogen bromide was next investigated and satisfactory results were achieved with this reagent at 0° in a mixture of chloroform and acetic anhydride. Some acid interchange took place under the conditions employed and the material was reesterified with diazomethane before isolation. The product obtained in this way melted at 183.5–185° and was identified as methyl 3(α)-acetoxy-11-keto-12-bromocholanate[2] (VII) by direct comparison with an authentic sample[3] prepared from methyl 3(α)-acetoxy-Δ^{11}-cholenate and hypobromous acid by the method of Ott and Reichstein (5).

Reesterification of the crude reaction mixture with methanolic hydrogen chloride instead of diazomethane resulted in methanolysis of the C_3-acetoxy group and gave methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII). This substance was oxidized with chromic acid to methyl 3,11-diketo-12-bromocholanate (XIX). Treatment of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) with zinc in acetic acid yielded methyl 3(α)-hydroxy-11-ketocholanate (XI) which, on hydrolysis, gave 3(α)-hydroxy-11-ketocholanic acid (X). Chromic acid converted X into 3,11-diketocholanic acid (XX), from which the methyl ester (XXI) was prepared. Compounds XVIII and XIX were identical with the corresponding products made from the sample of methyl 3(α)-acetoxy-11-hydroxy-12-bromocholanate which was prepared according to the synthesis of Ott and Reichstein (5).[3] It was further shown that XXI was identical with the product described by Lardon and Reichstein (6) and with a sample obtained by Long and Gallagher (7) in another series of reactions. In addition, the melting points and specific rotations of methyl 3(α)-acetoxy-11-ketocholanate (IX) and 3(α)-hydroxy-11-ketocholanic acid (X) are in close agreement with the values for these compounds which have recently been reported in the literature (6).

The separation of methyl 3(α)-acetoxy-11-keto-12-bromocholanate (VII) after treatment of methyl 3,9-epoxy-11-ketocholanate (V) with hydrogen bromide in acetic anhydride clearly establishes the position of the keto group in V. The latter compound may be obtained from methyl 3,9-epoxy-Δ^{11}-cholenate (I) in yields as high as 75 per cent by reworking the mother liquors (conversion to I and rebromination) and the procedure is therefore of considerable practical significance.

Although cleavage of the epoxide to a 3-bromo-9-hydroxy compound

[2] The configuration of VII will be discussed in Paper VII of this series.

[3] We are indebted to Dr. L. H. Sarett of Merck and Company for this sample.

is an obvious possibility, it was interesting to note that the product isolated in 89 per_cent yield was a derivative of 3(α)-hydroxy-12-bromocholanic acid.

The mechanism by which the bromine becomes attached at C_{12} is of some theoretical interest, and, although rigorous establishment of this mechanism is not possible from our experiments, certain observations may be mentioned.   Heilbron and associates (8) have reported the facile reduction of the tertiary halogen in 5,7-dibromo-6-ketocholestanyl acetate by hydrogen bromide.   This result suggests the possibility that methyl 3(α)-hydroxy-11-keto-9-bromocholanate may be formed at an intermediate stage in the cleavage and that this compound undergoes reduction by hydrogen bromide with subsequent rebromination at C_{12}.   Some evidence for the first step of such a mechanism was obtained in a study of the cleavage of methyl 3,9-epoxy-11-keto-12-bromocholanate (XVI) by hydrogen bromide in acetic anhydride.   When XVI was treated with hydrogen bromide, it was found that approximately 25 per cent of the theoretical amount of bromine was liberated, and about an equal amount of methyl 3(α)-acetoxy-11-keto-12-bromocholanate (VII) was isolated.   When the same experiment was carried out in the presence of hydrogen sulfide, added to remove the liberated bromine, the yield of methyl 3(α)-acetoxy-11-keto-12-bromocholanate (VII) was increased to 64 per cent.

The actual existence of a C_9-bromo derivative as a stable intermediate, however, is doubtful.   Although both the C_5 and C_7 monobromides have been obtained by bromination of 6-ketocholestanyl acetate (9), bromination of 11-keto derivatives leads exclusively to C_{12}-substituted products.   It has not been possible to obtain a 9,12-dibromo-11-ketone even in the presence of a large excess of bromine, whereas 5,7-dibromo-6-ketocholestanyl acetate is formed with comparative ease (8).

When C_{12} is unsubstituted, it seems unlikely that the mechanism which involves an intermediate at C_9 with reduction by hydrogen bromide to free bromine and subsequent bromination at position 12 can play any important part, for treatment of methyl 3,9-epoxy-11-ketocholanate with hydrogen bromide and acetic anhydride gave methyl 3(α)-acetoxy-11-keto-12-bromocholanate (VII) in 77 per cent yield, even in the presence of hydrogen sulfide.

An alternative mechanism, consistent with the experimental results, involves the enol of the 11-ketone.   This mechanism is outlined in Fig. 2.

When methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) was allowed to stand with aqueous methanolic potassium hydroxide for several hours, a mixture was obtained which, on esterification and chromatographic separation, yielded, in addition to unchanged starting material, considerable amounts of methyl 3,9-epoxy-11-ketocholanate (V).   The formation of the epoxide was not anticipated, but it can evidently result from a process

which is essentially a reversal of that outlined in Fig. 2. Conversion of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) into a 3,9-epoxy compound is an example of a change in structure which occurs in other closely related compounds.[4] When methyl 3(α)-hydroxy-11-keto-12-bromonorcholanate (XXVIII) (Fig. 3) was treated with hot aqueous potassium hydroxide, a large part of the starting material was converted to 3,9-epoxy-11-ketonorcholanic acid (XXIV). In addition, 3(α),12-dihydroxy-11-ketonorcholanic acid (XXIII) was formed. Potassium hydroxide in a boiling methanolic solution of XXVIII also yielded 3,9-epoxy-11-ketonorcholanic acid (XXIV) and, in addition, some 3(α)-
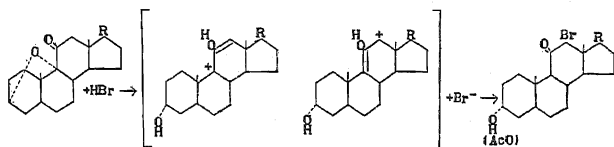
Fig. 2
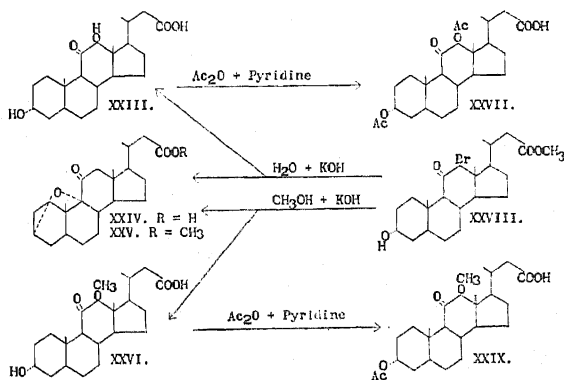
Fig. 3

hydroxy-11-keto-12-methoxynorcholanic acid (XXVI). It is apparent that methyl 3,11-diketo-12-bromocholanate (XXX) (Fig. 4) cannot form the 3,9-epoxide and on similar treatment the 3,11-diketo-12-hydroxy compound (XXXI) was obtained. The position of the hydroxyl group in XXXI was established by oxidation to methyl 3,11,12-triketonorcholanate (XXXII), which showed the characteristic spectrum of an α-diketone (4, 11, 12).

[4] In the period during which the present investigation was being carried out, both methyl 3(α)-hydroxy-11-ketocholanate and methyl 3,9-epoxy-11-ketocholanate were degraded to the corresponding etio acids by the method of Hoehn and Mason (10). This work made available a large number of intermediate compounds and will be reported in subsequent communications. The choice of the nor ester here is arbitrary.

Investigation of the cleavage of the epoxide grouping in various products derived from methyl 3,9-epoxy-11-ketocholanate by degradation of the side chain at C_{17} has revealed the interesting fact that when the length of the side chain is decreased the oxide ring becomes more resistant to cleavage with hydrogen bromide.   Methyl 3,9-epoxy-11-ketocholanate (V), 3,9-epoxy-11-ketobisnorcholanyldiphenylethylene, and methyl 3,9-epoxy-11-ketonorcholanate react with comparative ease and give the corresponding 3(α)-acetoxy-12-bromo-11-ketones in good yield.   Methyl 3,9-epoxy-11-ketobisnorcholanate and methyl 3,9-epoxy-11-ketoetiocholanate, however, require more drastic conditions and the main products are accompanied by appreciable amounts of dibromo derivatives.   Poor yields of unidentified crystalline products were obtained from 3,9-epoxy-11,20-pregnanedione and 3,9-epoxy-11-ketoetiocholanylmethyldiphenylethylene, although these cases appear to be complicated by other factors.   These latter products have not been further investigated.
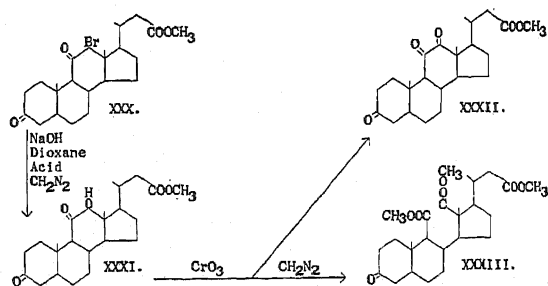
FIG. 4

An improved method for the preparation from desoxycholic acid of the essential intermediate methyl 3,9-epoxy-11,12-dibromocholanate (II) will be described in Paper VI.   From II by the steps described in the present paper methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) has been prepared in a yield of 81 per cent.   The yield of XVIII is given, since this compound may be used for the degradation of the side chain.

EXPERIMENTAL[5]

All melting points were determined on the Fisher-Johns apparatus.

Methyl 3,9-Epoxy-Δ^{11}-cholenate (I)—See (4).

Methyl 3,9-Epoxy-11,12-dibromocholanate (II and III)—See (4).

3,9-Epoxy-11-ketocholanic Acid (IV) from V—1.00 gm. of methyl 3,9-epoxy-11-ketocholanate (V) in 50 cc. of methanol and 5 cc. of 1 N aqueous sodium hydroxide were heated under a reflux condenser for 1 hour. The solution was diluted with water and acidified and the methanol was removed under reduced pressure. The precipitate which formed was washed with water and crystallized from acetone-water. The product (969 mg.) melted at 170–172°. Several recrystallizations raised the melting point to 174–174.5°. $[\alpha]_D = +90° \pm 2°$ (32.5 mg. in 3.00 cc. of chloroform).

$C_{24}H_{36}O_4$. Calculated, C 74.19, H 9.34; found, C 74.18, H 9.52

Methyl 3,9-Epoxy-11-ketocholanate (V) from XVI—19.2 gm. of methyl 3,9-epoxy-11-keto-12-bromocholanate (XVI) were dissolved in 20 cc. of benzene, 40 cc. of acetic acid were added, and the solution was cooled to 20°. 10 gm. of powdered zinc were added at such a rate that the temperature remained below 30° and the mixture was shaken frequently over a period of 45 minutes. The zinc was filtered from the solution and the filtrate was concentrated under reduced pressure. The residue was distributed between benzene and water and the organic phase was washed with water. The solvent was removed under reduced pressure and the residue crystallized from methanol to give 10.1 gm. of material which melted at 81–81.5°. Addition of water to the mother liquor gave two additional crops of crystals which weighed 4.80 gm. (m.p. 81.0–81.5°) and 0.68 gm. (m.p. 76–77°) and brought the total yield to 97 per cent. $[\alpha]_D = +88° \pm 2°$ (27.7 mg. in 3.00 cc. of chloroform).

$C_{25}H_{38}O_4$. Calculated, C 74.59, H 9.51; found, C 74.82, H 9.27

V from XVIII—2.00 gm. of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) were dissolved in 50 cc. of methanol to which 20 cc. of 25 per cent potassium hydroxide were added. After the solution had remained at room temperature overnight, it was diluted with a large volume of water, acidified with hydrochloric acid, and extracted with ether. The organic phase was washed with water, dried with sodium sulfate, concentrated to a small volume, and treated with diazomethane in ether. Chromatography of the oily mixture of esters on aluminum oxide gave 424 mg., eluted with benzene-petroleum ether 50:50 and 60:40. This material, when crystallized from methanol, gave a first crop of 250 mg. of material which melted at 77.5–78.5°. When the substance was mixed with methyl 3,9-epoxy-11-ketocholanate, the melting point was 78–79.5°

A further fraction eluted with benzene was crystallized from ether-petroleum ether and gave 120 mg. of material which melted at 144–148°. Recrystallization from the same solvents raised the melting point to 157.5–

158.5°.   The melting point was not depressed when the product was mixed with methyl 3(α)-hydroxy-11-keto-12-bromocholanate.

3(α)-Acetoxy-11-keto-12-bromocholanic Acid (VI) from XVIII—3.00 gm. of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) were dissolved in 60 cc. of acetic acid which was 1 N with hydrogen bromide. After the solution had remained at room temperature for 6 days, it was diluted with water to about 225 cc. and the crystals which separated were collected and washed with water.   The product (3.16 gm.) melted at 266–267°.   After several recrystallizations from chloroform-benzene, needles were obtained which melted at 274–275° (corrected) (taken with a glass disk heated on a metal bar). $[\alpha]_D = -5° \pm 2°$ (29.8 mg. in 4.00 cc. of chloroform).

$C_{26}H_{39}O_5Br$.   Calculated.   C 61.05, H 7.69, Br 15.62
                        Found.   " 60.97, " 7.69, " 15.57

Methyl 3(α)-Acetoxy-11-keto-12-bromocholanate (VII) from V—2.00 gm. of methyl 3,9-epoxy-11-ketocholanate (V) were placed in a pressure tube which contained 2.5 cc. of dry chloroform and 2.5 cc. of acetic anhydride.   The tube was cooled in an acetone-dry ice bath and 10 gm. of hydrogen bromide were added.   The gas condensed to form a dense viscous fluid with the contents of the tube.   After being sealed, the tube was placed in an ice bath and remained at 0° for 18 hours.   The tube was again cooled in a dry ice bath and opened.   The contents of the tube were poured on ice and chloroform was added.   The organic phase was washed with water until free of acetic acid.   Diazomethane in ether was added to restore any ester that had been hydrolyzed by the treatment.   The organic phase was washed with dilute acetic acid, with a solution of sodium carbonate and with water, and was dried with sodium sulfate.   The solution was concentrated to a small volume under reduced pressure, and after addition of methanol the solution was reduced again to a small volume and cooled. 1.98 gm. (76 per cent) of material which melted at 181.5–182.5° were obtained.   After four crystallizations from methanol the melting point was 183.5–185°.   There was no depression of the melting point when this material was mixed with an authentic sample[6] of methyl 3(α)-acetoxy-11-keto-12-bromocholanate. $[\alpha]_D = +8° \pm 2°$ (30.8 mg. in 3.00 cc. of acetone).

This compound also has been prepared by Ott and Reichstein (5). These authors reported $[\alpha]_D = +13° \pm 2°$.   It was found that samples made in this laboratory by oxidation of the 11,12-bromohydrin also had $[\alpha]_D = +13° \pm 2°$ to $+18° \pm 2°$.   However, reoxidation and subsequent crystallization lowered the value of $[\alpha]_D$ to $+8° \pm 2°$, which conforms

---

[6] Prepared by Dr. L. H. Sarett from methyl 3(α)-acetoxy-Δ^{11}-cholenate according to the method of Ott and Reichstein (5).

with the value found for VII prepared by cleavage of the 3,9-epoxide. The hydroxyl group at C_{11} apparently is not readily oxidized and a small amount of the hydroxyl compound would explain the $[\alpha]_D = +13°$ $\pm 2°$ previously reported for the bromoketone (5).

VII from IX—500 mg. of methyl 3(α)-acetoxy-11-ketocholanate (IX) were brominated in 10 cc. of acetic acid with 2.0 cc. of 1.32 N bromine in acetic acid in the presence of 1.0 cc. of 4.6 N hydrogen bromide in acetic acid. After 4.5 hours at room temperature a mixture of ether and chloroform was added and the organic phase was washed with water, dilute sodium hydroxide, water, and saturated sodium chloride and filtered through sodium sulfate. The solution was concentrated to a small volume under reduced pressure and diluted with petroleum ether. The crystals which formed (230 mg.) melted at 182–183°. After two recrystallizations from methanol the product melted at 185–185.5° and did not depress the melting point of VII prepared from V. $[\alpha]_D = +7° \pm 2°$ (36.3 mg. in 3.00 cc. of acetone).

VII from XVI—2.00 gm. of methyl 3,9-epoxy-11-keto-12-bromocholanate (XVI) in 2.50 cc. of chloroform and 2.50 cc. of acetic anhydride were treated at 0° with 10.0 gm. of dry hydrogen bromide for 17 hours according to the procedure described under VII from V. The solution was poured on ice and chloroform was added. Titration of aliquot portions of the chloroform and aqueous phases indicated the presence of 2.19 milliequivalents of bromine. The bromine was removed by washing with a solution of potassium iodide and sodium thiosulfate. The chloroform was washed with water until free of acetic acid and was then treated with a solution of diazomethane in ether. The organic phase was washed with dilute hydrochloric acid and with water and was evaporated to dryness. The residue was crystallized from methanol to give 610 mg. of material which melted at 145–150°. After three recrystallizations from methanol the material (243 mg.) had a constant melting point of 181–182° which was about 4° lower than the melting point of an authentic sample. To remove traces of halogen-containing compounds other than VII the product was treated in 10 cc. of acetic acid with 100 mg. of silver acetate on the steam bath for 1 hour. The solution was filtered, the filtrate was diluted with water, and the resulting crystals were collected, washed with water, and recrystallized from methanol. 161 mg. of material were obtained which melted at 182.5–184° and did not depress the melting point of an authentic sample of VII. $[\alpha]_D = +9° \pm 2°$ (31.1 mg. in 3.00 cc. of acetone).

VII from XVI in Presence of Hydrogen Sulfide—2.00 gm. of methyl 3,9-epoxy-11-keto-12-bromocholanate (XVI) were placed in a pressure tube to which were added 2.50 cc. of chloroform and 2.50 cc. of acetic anhydride. 1.0 gm. of hydrogen sulfide and 10.0 gm. of hydrogen bromide

were condensed in the tube at dry ice temperature and the tube was sealed and placed in an ice bath. After 17 hours the tube was cooled in a dry ice bath and opened. The contents were poured on ice. Chloroform was added and the organic phase was washed with water. The aqueous phase contained the equivalent of 6.2 cc. of 1 N hydrogen sulfide. The organic phase was evaporated to dryness under reduced pressure, and the residue was treated with a solution of diazomethane in ether. The ether was removed under reduced pressure and the residue crystallized from petroleum ether to give 2.23 gm. of product which melted at 100–148° and had a very pungent odor. To remove impurities formed from the hydrogen sulfide the material was treated in 125 cc. of methanol with 1.0 gm. of silver acetate. After 18 hours at room temperature a few drops of saturated ammonium sulfate were added to coagulate the silver sulfide which had formed, and the solution was filtered. The filtrate was evaporated to dryness under reduced pressure, the residue was distributed between benzene and water, and the benzene solution was washed with water and evaporated to dryness. The residue was treated with 5.0 cc. of pyridine and 5.0 cc. of acetic anhydride at room temperature. After 18 hours the solution was seeded and diluted with ice water and the resulting crystals were collected and washed with water. The crude product was crystallized from methanol to give 1.40 gm. (64 per cent) of material which melted at 182–184°. After recrystallization from methanol the product melted at 184–185° and did not depress the melting point of VII obtained from V. $[\alpha]_D = +8° \pm 2°$ (31.3 mg. in 3.00 cc. of acetone).

VII from V in Presence of Hydrogen Sulfide—2.00 gm. of methyl 3,9-epoxy-11-ketocholanate (V) were treated with hydrogen bromide at 0° in the presence of chloroform, acetic anhydride, and hydrogen sulfide, as described in the preceding paragraph. 1.87 gm. of material which melted at 184–185° and did not depress the melting point of an authentic sample of VII were obtained. When the residue of the mother liquor was crystallized from ligroin, an additional 136 mg. of material which melted at 182.5–184° were separated. The total yield was 77 per cent. $[\alpha]_D = +8° \pm 2°$ (30.0 mg. in 3.00 cc. of acetone).

VII from XVIII—4.84 gm. of methyl 3(α)-hydroxy-11-keto-12-bromo-cholanate (XVIII) were acetylated with 10 cc. of pyridine and 10 cc. of acetic anhydride. After 24 hours at room temperature a small amount of ice and water was added. The crystals which separated (5.23 gm.) melted at 183.5–184.5°. After several recrystallizations from methanol a product was obtained which melted at 185–185.5°. $[\alpha]_D = +7° \pm 2°$ (36.3 mg. in 3.00 cc. of acetone).

$C_{27}H_{41}O_5Br$.   Calculated.   C 61.70, H 7.86, Br 15.22
            Found.   " 61.40, " 7.76, " 15.42

3(α)-Acetoxy-11-ketocholanic Acid (VIII) from VI—1.02 gm. of 3(α)-acetoxy-11-keto-12-bromocholanic acid (VI) in 30 cc. of acetic acid were treated with 1.0 gm. of powdered zinc on the steam bath for 20 minutes. The solution was filtered and evaporated to dryness under reduced pressure. The residue was distributed between benzene and water and the benzene phase was washed with water, filtered through sodium sulfate, concentrated under reduced pressure, and diluted with ligroin. The crystals which formed (732 mg.) melted at 227–229°. A second crop of 45 mg. which melted at 223–224° was obtained from the filtrate. The melting point of the first crop was not raised by repeated recrystallizations from benzene-ligroin. $[\alpha]_D = +77° \pm 2°$ (31.4 mg. in 6.00 cc. of methanol).

$C_{26}H_{40}O_5$. Calculated, C 72.19, H 9.32; found, C 72.39, H 9.25

Methyl 3(α)-Acetoxy-11-ketocholanate (IX) from VII—526 mg. of methyl 3(α)-acetoxy-11-keto-12-bromocholanate (VII) in 10 cc. of acetic acid were treated with 500 mg. of powdered zinc as described under VIII from VI. The residue was crystallized from acetone-water to give 391 mg. of product which melted at 132.5–133.5°. The filtrate yielded a second crop of 30 mg. which melted at 128–130°. A sample purified by recrystallization from acetone-water melted at 134–134.5°. $[\alpha]_D = +68° \pm 2°$ (34.6 mg. in 3.00 cc. of acetone) (6).

$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 72.88, H 9.22

3(α)-Hydroxy-11-ketocholanic Acid (X) from XI—2.71 gm. of methyl 3(α)-hydroxy-11-ketocholanate (XI) were hydrolyzed in 25 cc. of methanol with 3.0 cc. of 5 N aqueous sodium hydroxide. The solution was refluxed for 30 minutes and was diluted with water. The methanol was removed under reduced pressure and the solution was acidified with hydrochloric acid. The precipitate was collected, washed with water, and recrystallized from acetone-water to give 2.55 gm. of material which melted at 223–224°. Repeated recrystallizations did not raise the melting point. $[\alpha]_D = +61° \pm 2°$ (31.5 mg. in 3.00 cc. of methanol) (6).

$C_{24}H_{38}O_4$. Calculated, C 73.80, H 9.81; found, C 73.90, H 9.79

Methyl 3(α)-Hydroxy-11-ketocholanate (XI) from XVIII—4.84 gm. of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) were dissolved in 25 cc. of acetic acid, the solution was cooled to 20°, and 2.5 gm. of powdered zinc were added in small portions. The temperature was maintained below 25° to avoid acetylation of the C_3-hydroxyl group. After 30 minutes the solution was filtered, the filtrate was concentrated to dryness under reduced pressure, and the residue was distributed between benzene and water. The organic phase was washed with water. The bromide ion in

the aqueous phase was 97 per cent of the theoretical amount.   The benzene solution was evaporated to a small volume and diluted with petroleum ether.   The crystals which formed (3.60 gm.) melted at 101.5–102.5°.   A second crop of 140 mg. which melted at 100–101° was obtained.   Recrystallization from ether-petroleum ether raised the melting point to 102.5–103°. $[\alpha]_D = +62° \pm 2°$ (30.6 mg. in 3.00 cc. of methanol).

$C_{25}H_{40}O_4$.   Calculated, C 74.21, H 9.97; found, C 73.99, H 9.84

3(α),11-Dihydroxycholanic Acid (XII) from X—390 mg. of 3(α)-hydroxy-11-ketocholanic acid (X) were reduced with hydrogen in 15 cc. of acetic acid in the presence of 50 mg. of platinum oxide.   After 11.5 hours 0.98 molar equivalents of hydrogen had been absorbed.   The platinum was filtered from solution and the solvent was removed under reduced pressure. The residue was dissolved in 20 cc. of methanol, 5 cc. of 5 N aqueous sodium hydroxide were added, and the solution was heated on the steam bath for a short time.   The methanol was removed under reduced pressure, the residue was dissolved in water, and the solution was acidified with acetic acid.   The resulting precipitate was filtered from solution, washed with water, dried, and recrystallized from benzene.   The product (203 mg.) melted at 194–196°.   After several recrystallizations the melting point was raised to 199.5–200.5°. $[\alpha]_D = +55° \pm 2°$ (30.0 mg. in 3.00 cc. of methanol).

$C_{24}H_{40}O_4$.   Calculated, C 73.43, H 10.27; found, C 73.37, H 10.11

Methyl 3(α),11-Dihydroxycholanate (XIII) from XII—392 mg. of 3(α),11-dihydroxycholanic acid (XII) were esterified with diazomethane in ether.   The ester failed to crystallize from dilute methanol or acetone or from mixtures of petroleum ether and other solvents.   Chromatographic separation on aluminum oxide yielded fractions with benzene and petroleum ether 3:1 and benzene alone.   All of these fractions failed to crystallize. A granular product which separated from a benzene solution of the chromatographed ester, after addition of petroleum ether, was not visibly crystalline.   The material melted at 99–101°. $[\alpha]_D = +49° \pm 2°$ (30.1 mg. in 3.00 cc. of acetone).

$C_{25}H_{42}O_4$.   Calculated, C 73.84, H 10.41; found, C 73.87, H 10.48

Methyl 3,9-Epoxy-11-acetoxy-12-bromocholanate (XIV) from II—See (4).

XIV from XV—400 mg. of methyl 3,9-epoxy-11-hydroxy-12-bromocholanate (XV) were dissolved in a mixture of 2 cc. of benzene and 10 cc. of 0.2 N sulfuric acid (13) in acetic acid.   3 cc. of acetic anhydride were added slowly while the solution was cooled.   After the mixture had stood at room temperature for 18 hours, ice water was added and the solution was extracted with benzene.   The benzene solution was washed with water, with sodium carbonate solution, and with water and evaporated to dryness

under reduced pressure. The residue crystallized from acetone-water to give 334 mg. of material which melted at 131–132°. After recrystallization from acetone-water the product melted at 133.5–134.5° and did not depress the melting point of methyl 3,9-epoxy-11-acetoxy-12-bromocholanate (4). $[\alpha]_D = +16° \pm 2°$ (30.1 mg. in 3.00 cc. of chloroform). A sample of methyl 3,9-epoxy-11-hydroxy-12-bromocholanate (XV) was recovered unchanged in a yield of 76 per cent after treatment at room temperature for 23 hours with acetic anhydride and pyridine.

Methyl 3,9-Epoxy-11-hydroxy-12-bromocholanate (XV) from II. (a) With Silver Oxide—1.09 gm. of methyl 3,9-epoxy-11,12-dibromocholanate (II) (4), 80 cc. of acetone, 20 cc. of water, 1.39 gm. of silver oxide, and 10 gm. of glass beads were shaken in a glass-stoppered bottle for 30 minutes. The solution was filtered, concentrated to about 15 cc., and distributed between water and benzene. The organic phase was washed with water, dried with sodium sulfate, and concentrated to a small volume. After addition of petroleum ether, crystals separated (543 mg.) which melted at 184.5–185.5°. $[\alpha]_D = +58° \pm 2°$ (30.5 mg. in 3.00 cc. of chloroform).

$C_{25}H_{39}O_4Br$. Calculated, C 62.10, H 8.13; found, C 62.36, H 8.24

(b) *With Silver Carbonate*—16.4 gm. of methyl 3,9-epoxy-11,12-dibromocholanate (II) (4), 240 cc. of acetone, 10 cc. of water, 4.56 gm. of silver carbonate, and 20 gm. of glass beads in a glass-stoppered flask were shaken for 3 hours. The solution was filtered and evaporated to dryness under reduced pressure. The residue crystallized from benzene-petroleum ether to give 7.56 gm. of product which melted at 182.5–184°. After several recrystallizations from benzene-petroleum ether the melting point was raised to 186–187°. Admixture with a sample prepared as under (a) did not depress the melting point. $[\alpha]_D = +58° \pm 2°$ (31.8 mg. in 3.00 cc. of chloroform).

Methyl 3,9-Epoxy-11-keto-12-bromocholanate (XVI) from II—87.6 gm. of methyl 3,9-epoxy-11,12-dibromocholanate (II) (m.p. 141.5–143°) (4) were suspended in 2100 cc. of acetone contained in a flask which was placed in a water bath at about 25°. 40 gm. of silver chromate and a solution of 31 gm. of chromic acid in 200 cc. of water were added and the solution was stirred mechanically for 2 hours. A large amount of heat was produced but the temperature within the flask was maintained at approximately 25–28°. After 2 hours all crystals of the starting material had disappeared from suspension. With continued mechanical stirring 77 cc. of 5 N sulfuric acid were added and after 30 minutes the solution was filtered and concentrated under reduced pressure. The first crop of crystals which separated weighed 48.4 gm. and melted at 114–115°. The second crop, obtained after further concentration of the filtrate, weighed 23.9 gm. and melted at 112–114°. The total yield was 94 per cent of the theoretical

amount.   A sample purified by recrystallization from acetone-water melted
at 114.5–115°. $[\alpha]_D = -36° \pm 2°$ (30.0 mg. in 3.00 cc. of chloroform).

C$_{25}$H$_{37}$O$_4$Br.   Calculated.   C 62.36, H 7.74, Br 16.6
                            Found.   " 62.45, " 7.82, " 16.4

XVI from V—403 mg. of methyl 3,9-epoxy-11-ketocholanate (V) were
brominated in 20 cc. of 0.1 N hydrogen bromide in acetic acid with 2.5 cc.
of 1.0 N bromine in acetic acid.   After 19 hours water was added and the
crystals which separated were washed with water and recrystallized from
acetone-water.   The product (81 per cent of the theoretical amount)
melted at 112–113° and after crystallization from acetone-water the melting
point was 114–115°.   The melting point was not depressed when the
product was mixed with an authentic sample of XVI.

XVI from XV—484 mg. of methyl 3,9-epoxy-11-hydroxy-12-bromo-
cholanate (XV) were oxidized in a mixture of 5 cc. of chloroform and 20
cc. of acetic acid with 3.30 cc. of 1.82 N chromic acid in 95 per cent acetic
acid.   After 2 hours at room temperature the solution was diluted with
water and extracted with chloroform.   The chloroform solution was
washed with water and concentrated to dryness under reduced pressure.
The residue was crystallized from acetone-water to give 348 mg. of material
which melted at 115–116° and did not depress the melting point of XVI
obtained from II.   Addition of water to the filtrate yielded a second crop
of crystals (62 mg.) which melted at 114–115° and raised the total yield to
85 per cent. $[\alpha]_D = -36° \pm 2°$ (30.6 mg. in 3.00 cc. of chloroform).

3(α)-Hydroxy-11-keto-12-bromocholanic Acid (XVII) from XVIII—484
mg. of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) were
dissolved in 20 cc. of methanol to which were added 1.0 cc. of water and
1.20 cc. of 1.25 N methanolic sodium hydroxide.   After the solution had
stood at room temperature for 16 hours, it was diluted with water to tur-
bidity.   Crystals formed, indicating that hydrolysis of the ester was not
complete.   The mixture was heated on the steam bath until the crystals
dissolved (45 minutes).   Then the solution was diluted with water and the
methanol was removed under reduced pressure.   The solution was acidified
with sulfuric acid and the precipitate was filtered from solution.   The
filtrate contained 10.5 per cent of the theoretical amount of bromide ion.
The precipitated acid was recrystallized from acetone-water to give 380 mg.
of material which melted at 177–178° (with decomposition).   After two
recrystallizations from acetone-benzene and one crystallization from
methanol-water a product was obtained which melted at 189–190° (with
effervescence). $[\alpha]_D = -5° \pm 2°$ (29.5 mg. in 3.00 cc. of methanol).

C$_{24}$H$_{37}$O$_4$Br.   Calculated, C 61.40, H 7.95; found, C 61.06, H 8.09

Methyl 3(α)-Hydroxy-11-keto-12-bromocholanate (XVIII) from V—20.0
gm. of methyl 3,9-epoxy-11-ketocholanate (V), 25 cc. of chloroform, and

25 cc. of acetic anhydride were placed in a pressure bottle which was cooled in a dry ice-acetone bath. 100 gm. of dry hydrogen bromide were added, and the bottle was sealed and placed in an ice bath. After 16 hours the bottle was cooled in a dry ice bath, opened, and the contents were poured on ice. Chloroform was added and the organic phase was washed with water and concentrated under reduced pressure to about 125 cc. 250 cc. of methanol and 40 cc. of 2.0 N methanolic hydrogen chloride were added to the solution. After 20 hours at room temperature the solution was concentrated under reduced pressure and benzene was added. The benzene solution was washed with water, with sodium carbonate solution, and with water, and was filtered through sodium sulfate. After concentration under reduced pressure to about 75 cc., ligroin was added. The crystals which formed (18.3 gm.) melted at 157.5–158°. From the filtrate two additional crops of 2.32 gm. and 0.12 gm., which melted at 156–157° and 155–156° respectively, were obtained. The yield was 86 per cent. The mother liquor was retreated with hydrogen bromide as described and 0.71 gm. of material which melted at 154–155° was obtained. This raised the total yield to 89 per cent. Several recrystallizations of the first crop from benzene-ligroin did not alter the melting point. The melting point was not depressed when the product was mixed with a sample of methyl 3(α)-hydroxy-11-keto-12-bromocholanate, $[\alpha]_D = -5° \pm 2°$ (23.7 mg. in 3.00 cc. of methanol), which was made from methyl 3(α)-acetoxy-11-keto-12-bromocholanate described in the second paragraph of section VII from V. $[\alpha]_D = -5° \pm 2°$ (30.8 mg. in 3.00 cc. of methanol).

$C_{25}H_{39}O_4Br$. Calculated. C 62.10, H 8.13, Br 16.53
Found.   " 61.85, " 8.28, " 16.51

Methyl 3,11-Diketo-12-bromocholanate (XIX) from XVIII[7]—150 mg. of methyl 3(α)-hydroxy-11-keto-12-bromocholanate (XVIII) were oxidized in 15.5 cc. of acetic acid with 0.50 cc. of 1.88 N chromic acid in 95 per cent acetic acid at room temperature. After 1 hour the solution was diluted with water and the resulting precipitate was collected and washed with water. After crystallization from methanol-water 122 mg. of material which melted at 160–163° were obtained. After three further recrystallizations the product melted at 161–163°. $[\alpha]_D = -20° \pm 2°$ (30.1 mg. in 3.00 cc. of acetone).

$C_{25}H_{37}O_4Br$. Calculated. C 62.02, H 7.51, Br 16.54
Found.   " 62.36, " 7.75, " 16.60

A sample of methyl 3(α)-hydroxy-11-keto-12-bromocholanate derived from methyl 3(α)-acetoxy-11-hydroxy-12-bromocholanate (5)[8] prepared

as described under VII from V was oxidized to the 3,11-diketone.[7] The product melted at 161–163° and did not depress the melting point of the sample described in the preceding paragraph. $[\alpha]_D = -18° \pm 2°$ (30.4 mg. in 3.00 cc. of acetone).

3,11-Diketocholanic Acid (*XX*) *from X*—1.95 gm. of 3(α)-hydroxy-11-ketocholanic acid (X) were oxidized at room temperature in 10 cc. of chloroform and 40 cc. of acetic acid with 8.1 cc. of 1.85 N chromic acid in 95 per cent acetic acid. After 2 hours benzene was added and the organic phase was washed with water until free from acetic acid. The solvent was removed under reduced pressure and the residue was crystallized from acetone-water to give 1.51 gm. of material which melted at 173.5–175.5°. After several recrystallizations from acetone-water the melting point was 176–177°. $[\alpha]_D = +63° \pm 2°$ (28.6 mg. in 3.00 cc. of acetone).

$C_{24}H_{36}O_4$. Calculated, C 74.19, H 9.34; found, C 74.00, H 9.15

Methyl 3,11-Diketocholanate (*XXI*) *from XX*—498 mg. of 3,11-diketo-cholanic acid (XX) were esterified with a solution of diazomethane in ether and the ester was crystallized from petroleum ether. After several recrystallizations from petroleum ether the ester melted at 85–86°. The melting point was not depressed when the product was mixed with a sample of XXI, kindly furnished by Dr. T. F. Gallagher (7). $[\alpha]_D = +61° \pm 1°$ (40.0 mg. in 4 cc. of acetone) (6).

$C_{25}H_{38}O_4$. Calculated, C 74.58, H 9.51; found, C 74.75, H 9.74

Methyl 3(α)-Acetoxy-11-hydroxycholanate (*XXII*) *from XIII*—100 mg. of methyl 3(α),11-dihydroxycholanate (XIII) were dissolved in 0.60 cc. of pyridine and acetylated with 0.50 cc. of acetic anhydride at 70°. After 1 hour ether and water were added, the ether solution was washed with dilute hydrochloric acid and with water, and the solvent was dried with sodium sulfate and evaporated. The residue was crystallized from a small volume of ether-petroleum ether. 78 mg. which melted at 144–147° were obtained. After recrystallization in the same manner the melting point was 147–148°. $[\alpha]_D = +69° \pm 2°$ (30.1 mg. in 3.00 cc. of acetone (5, 6)).

3(α),12-Dihydroxy-11-ketonorcholanic Acid (*XXIII*) *from XXVIII*—4.20 gm. of methyl 3(α)-hydroxy-11-keto-12-bromonorcholanate (XXVIII) were added to 100 cc. of 10 per cent aqueous potassium hydroxide and the suspension was heated under a reflux condenser for 2 hours. The mixture was acidified with hydrochloric acid and extracted with ether. The ether solution was washed with water until neutral to litmus, filtered through sodium sulfate, and concentrated to a small volume. The residue was dissolved in benzene and the solution was concentrated. 880 mg. of crystals which melted at 222–225° were obtained and a second crop (100

mg.) which melted at 201–210° formed in the mother liquor. (Additional material was obtained from the mother liquor; see XXIV from XXVIII.) The analytic sample was recrystallized from ethyl acetate-ligroin. When heated slowly, the crystals melted at 214–216°; on rapid heating the melting point was 226–228°. $[\alpha]_D = +61° \pm 2°$ (30.0 mg. in 3.00 cc. of methanol).

$C_{23}H_{36}O_5$. Calculated, C 70.37, H 9.25; found, C 70.41, H 9.36

3,9-Epoxy-11-ketonorcholanic Acid (XXIV) from XXVIII in Aqueous Alkali—The filtrate of XXIII from XXVIII yielded two additional crops of crystals which weighed 760 mg. (m.p. 110–115°) and 819 mg. (m.p. 112–130°). The two crops were combined and recrystallized, first from benzene and then from chloroform-petroleum ether. The recrystallized material melted at 158–160° and did not depress the melting point of 3,9-epoxy-11-ketonorcholanic acid.

XXIV from XXVIII in Methanolic Alkali—The 0.84 gm. of material described under XXVI from XXVIII melted at 120–130°. After recrystallization from benzene and then from chloroform-petroleum ether a product was obtained which melted at 160–161° and did not depress the melting point of 3,9-epoxy-11-ketonorcholanic acid.

Methyl 3,9-Epoxy-11-ketonorcholanate (XXV) from XXIV—A portion of the acid described in XXIV from XXVIII in aqueous alkali was esterified with diazomethane; the ester, crystallized from methanol, melted at 122–123° and did not depress the melting point of methyl 3,9-epoxy-11-ketonorcholanate. The preparation of XXIV from methyl 3,9-epoxy-11-keto-12-bromocholanate through oxidation of the diphenyl ethylene derivative will be described in a later paper.

3(α)-Hydroxy-11-keto-12-methoxynorcholanic Acid (XXVI) from XXVIII—3.00 gm. of methyl 3(α)-hydroxy-11-keto-12-bromonorcholanate (XXVIII) were dissolved in 80 cc. of methanol which contained 5 cc. of 50 per cent aqueous potassium hydroxide and the solution was refluxed for 2.5 hours. The solution was cooled, diluted with water, and extracted with ether. The aqueous phase was acidified with dilute hydrochloric acid and extracted with ether. The ether layer was washed with water, filtered through sodium sulfate, concentrated to about 30 cc., and diluted with petroleum ether. 1.40 gm. of crystals which melted at 220–230° were separated. (Evaporation of the mother liquor and crystallization of the residue from chloroform-petroleum ether gave 0.84 gm. of material. This product is described under XXIV from XXVIII in methanolic alkali.) After recrystallization from methanol-water 690 mg. of material which melted at 245–246° were obtained. $[\alpha]_D = +80° \pm 2°$ (28.1 mg. in 3.00 cc. of methanol).

$C_{24}H_{38}O_5$. Calculated, C 70.90, H 9.42; found, C 70.70, H 9.37

3(α),12-Diacetoxy-11-ketonorcholanic Acid (XXVII) from XXIII—647 mg. of 3(α),12-dihydroxy-11-ketonorcholanic acid (XXIII) were dissolved in 3 cc. of pyridine and 3 cc. of acetic anhydride. After the solution had stood overnight at room temperature, ice was added, and the mixture was taken up in ether-chloroform and washed with water, with dilute hydrochloric acid, and then with water. The organic phase was filtered through sodium sulfate, concentrated to a small volume, and diluted with petroleum ether. 613 mg. of crystals which melted at 237–238.5° were obtained. After several recrystallizations from chloroform-petroleum ether the melting point was constant at 242.5–243.5°. $[\alpha]_D = +124° \pm 2°$ (26.0 mg. in 3.00 cc. of chloroform).

$C_{27}H_{40}O_7$. Calculated, C 68.04, H 8.46; found, C 68.00, H 8.26

Methyl 3(α)-Hydroxy-11-keto-12-bromonorcholanate (XXVIII)—This was obtained from the 3,9-epoxy-11-ketocholanic acid derivative by degradation of the side chain and subsequent cleavage of the 3,9-epoxide with hydrogen bromide. Preparation of this compound will be described in a later paper.

3(α)-Acetoxy-11-keto-12-methoxynorcholanic Acid (XXIX) from XXVI—332 mg. of 3(α)-hydroxy-11-keto-12-methoxynorcholanic acid (XXVI) were dissolved in 3 cc. of pyridine and 3 cc. of acetic anhydride. After the solution had remained overnight, ice and a mixture of ether and chloroform were added and the organic phase was washed with water, dilute hydrochloric acid, and water and filtered through sodium sulfate. The filtrate was concentrated to a small volume under reduced pressure and diluted with petroleum ether. The crystals which formed (185 mg.) melted at 232–235°. Several recrystallizations from chloroform-petroleum ether raised the melting point to 240–240.5°. $[\alpha]_D = +93° \pm 2°$ (24.6 mg. in 3.00 cc. of chloroform).

$C_{26}H_{40}O_6$. Calculated, C 69.61, H 8.99; found, C 69.37, H 8.76

Methyl 3,11-Diketo-12-bromonorcholanate (XXX) from XXVIII—10.00 gm. of methyl 3(α)-hydroxy-11-keto-12-bromonorcholanate (XXVIII) in 30 cc. of acetic acid were oxidized with 25 cc. of 2.04 N chromic acid in 95 per cent acetic acid. After 3 hours at room temperature water was added and the resulting mixture was extracted with chloroform-ether. The organic phase was washed with water, dilute sodium hydroxide, water, and saturated sodium chloride solution and was dried with sodium sulfate. After concentration to a small volume addition of petroleum ether caused separation of crystals. The first crop melted at 160–161.5° and weighed 7.47 gm. 560 mg. which separated from the mother liquor melted at 158–161°. The analytic sample was prepared by crystallization several

times from chloroform-petroleum ether. M.p. 161–162.5°. $[\alpha]_D = -29°$ ± 2° (31.8 mg. in 3.00 cc. of chloroform).

$C_{24}H_{35}O_4Br$. Calculated. C 61.66, H 7.55, Br 17.10
Found.   " 61.36, " 7.54, " 17.54

Methyl 3,11-Diketo-12-hydroxynorcholanate (XXXI) from XXX—7.46 gm. of methyl 3,11-diketo-12-bromonorcholanate (XXX) were dissolved in 50 cc. of dioxane, 100 cc. of 0.5 N aqueous sodium hydroxide were added, and the mixture was heated on the steam bath for 3 hours. The solution was cooled and extracted with ether. The aqueous phase was acidified with dilute hydrochloric acid and extracted with ether. The ether solution was washed with water and treated with a solution of diazomethane in ether. The excess diazomethane was removed on the steam bath, chloroform was added, and the solution was washed with dilute sodium hydroxide, water, and saturated salt solution and filtered through sodium sulfate. When the filtrate was concentrated and diluted with petroleum ether, 4.37 gm. of crystals which melted at 195–198° were obtained. After several recrystallizations from chloroform-petroleum ether the melting point was raised to 200–201.5°. $[\alpha]_D = +68° ± 2°$ (30.8 mg. in 3.00 cc. of chloroform).

$C_{24}H_{36}O_5$. Calculated, C 71.25, H 8.97; found, C 71.01, H 9.12

Methyl 3,11,12-Triketonorcholanate (XXXII) from XXXI—1.50 gm. of methyl 3,11-diketo-12-hydroxynorcholanate (XXXI) were oxidized in 30 cc. of purified acetic acid and 10 cc. of alcohol-free chloroform with 5.00 cc. of 1.98 N chromic acid in 95 per cent acetic acid. The solution was cooled in an ice bath before addition of the chromic acid and remained at 5° overnight. Water and ether were added and the organic phase was washed well with water and then with dilute sodium hydroxide. (See XXXIII from XXXI for treatment of the alkaline extract.) The neutral phase, after the alkaline extraction, was washed with water and with saturated salt solution and filtered through sodium sulfate. The solvent was removed under reduced pressure and the residue was crystallized from ether-petroleum ether to give 892 mg. of material which melted at 148–151°. Recrystallization of the product from ether-petroleum ether raised the melting point to 152–154°. The purified material gave a negative enol test with ferric chloride. $[\alpha]_D = +117° ± 6°$ (10.4 mg. in 3.00 cc. of chloroform). The absorption spectrum in 95 per cent ethanol showed maxima at 282 mμ (log ε = 2.22) and at 355 mμ (log ε = 1.74).

$C_{24}H_{34}O_5$. Calculated, C 71.61, H 8.51; found, C 71.68, H 8.24

Trimethyl 3-Keto-11‖12-norcholanate (XXXIII) from XXXI—The alkaline extracts of the organic phase described under XXXII from XXXI

were combined, acidified with dilute hydrochloric acid, and extracted with ether. The ether solution was washed with water, filtered through sodium sulfate, and concentrated to dryness under reduced pressure. The oily residue (332 mg.) was esterified with diazomethane in ether, the excess diazomethane was removed on the steam bath, and the solution was washed with dilute sodium hydroxide and water and filtered through sodium sulfate. When the filtrate was concentrated and diluted with petroleum ether, crystals formed. After recrystallization from ether-petroleum ether the product melted at 141–141.5°. $[\alpha]_D = +37° \pm 2°$ (28.1 mg. in 3.00 cc. of chloroform).

$$C_{26}H_{40}O_7. \quad \text{Calculated.} \quad \text{C } 67.21, \text{ H } 8.68$$
$$\text{Found.} \quad \text{`` } 67.61, \text{ `` } 8.51$$
$$\text{`` } 67.35, \text{ `` } 8.43$$

SUMMARY

A method is described for the conversion of methyl 3,9-epoxy-11,12-dibromocholanate into $3(\alpha)$-hydroxy-11-ketocholanic acid. Silver oxide converts methyl 3,9-epoxy-11,12-dibromocholanate (m.p. 143°) into the 11-hydroxy-12-bromo derivative which can be oxidized to a bromoketone. Debromination with zinc in acetic acid yields methyl 3,9-epoxy-11-keto-cholanate. The 3,9-cyclic ether in the compound last mentioned is ruptured by treatment at 0° with a high concentration of hydrogen bromide in acetic anhydride and chloroform to give methyl $3(\alpha)$-acetoxy-11-keto-12-bromocholanate, which in turn can be debrominated with zinc and hydrolyzed with alkali to yield $3(\alpha)$-hydroxy-11-ketocholanic acid. The following derivatives have been prepared: $3(\alpha)$-acetoxy-11-ketocholanic acid and methyl ester; $3(\alpha)$-hydroxy-11-keto-12-bromocholanic acid and methyl ester; methyl 3,11-diketo-12-bromocholanate; 3,11-diketocholanic acid and methyl ester; $3(\alpha),11$-dihydroxycholanic acid and methyl ester; and methyl $3(\alpha)$-acetoxy-11-hydroxycholanate.

Methyl $3(\alpha)$-hydroxy-11-keto-12-bromocholanate in aqueous methanolic potassium hydroxide lost hydrogen bromide and was converted in part into 3,9-epoxy-11-ketocholanic acid by a rearrangement which was essentially the reverse of the process by which the 3,9-cyclic ether was ruptured through addition of hydrogen bromide.

Methyl $3(\alpha)$-hydroxy-11-keto-12-bromonorcholanate, when treated with aqueous potassium hydroxide, gave a mixture from which 3,9-epoxy-11-ketonorcholanic acid and $3(\alpha),12$-dihydroxy-11-ketonorcholanic acid were isolated. Methyl $3(\alpha)$-hydroxy-11-keto-12-bromonorcholanate in methanolic potassium hydroxide yielded the 3,9-epoxy derivative and $3(\alpha)$-hydroxy-11-keto-12-methoxynorcholanic acid.

Methyl 3,11-diketo-12-bromonorcholanate with sodium hydroxide

yielded the 3,11-diketo-12-hydroxy derivative which was oxidized to the 3,11,12-triketo compound and a 3-ketotricarboxylic acid following rupture of Ring C.

The conditions and various factors which influence cleavage of the 3,9-epoxy structure are given and a probable mechanism of reaction to explain attachment of bromine at C_{12} and not C_9 as a result of the rupture of the cyclic ether is discussed.

BIBLIOGRAPHY

1. Engel, L. L., Mattox, V. R., McKenzie, B. F., McGuckin, W. F., and Kendall, E. C., *J. Biol. Chem.*, **162**, 565 (1946).
2. Turner, R. B., Mattox, V. R., Engel, L. L., McKenzie, B. F., and Kendall, E. C., *J. Biol. Chem.*, **162**, 571 (1946).
3. Sarett, L. H., *J. Biol. Chem.*, **162**, 591 (1946).
4. Mattox, V. R., Turner, R. B., Engel, L. L., McKenzie, B. F., McGuckin, W. F., and Kendall, E. C., *J. Biol. Chem.*, **164**, 569 (1946).
5. Ott, G. H., and Reichstein, T., *Helv. chim. acta*, **26**, 1799 (1943).
6. Lardon, A., and Reichstein, T., *Helv. chim. acta*, **26**, 586 (1943).
7. Long, W. P., and Gallagher, T. F., *J. Biol. Chem.*, **162**, 511 (1946).
8. Heilbron, I. M., Jackson, H., Jones, E. R. H., and Spring, F. S., *J. Chem. Soc.*, 102 (1938).
9. Heilbron, I. M., Jones, E. R. H., and Spring, F. S., *J. Chem. Soc.*, 801 (1937).
10. Hoehn, W. M., and Mason, H. L., *J. Am. Chem. Soc.*, **62**, 569 (1940).
11. Barnett, J., and Reichstein, T., *Helv. chim. acta*, **21**, 926 (1938).
12. Wintersteiner, O., and Moore, M., *J. Biol. Chem.*, **162**, 725 (1946).
13. Conant, J. B., and Bramann, G. M., *J. Am. Chem. Soc.*, **50**, 2305 (1928).

ON THE MECHANISM OF THE ANAEROBIC SYNTHESIS
OF ACETYLCHOLINE*

By M. A. LIPTON and E. S. GUZMAN BARRON

(From the Chemical Division, Department of Medicine, University of Chicago, Chicago)

A recent review by Feldberg (1) summarizes the results of other workers dealing with the synthesis of acetylcholine *in vitro*. It is known that acetylcholine can be synthesized by respiring brain tissue in oxygen in the presence of glucose, lactate, or pyruvate (2–4) or by homogenized brain or brain extracts anaerobically in the presence of added adenosine triphosphate (ATP) (5, 6). The synthesis is accelerated by K^+ and inhibited by Ca^{++} (7). The enzyme system appears to require active sulfhydryl groups because air, iodoacetate, iodine, cystine, and Cu^{++} inhibit, while cysteine (7) augments the activity of preparations which have been partially inactivated by dialysis. ATP is specific for the synthesis, since neither adenosine diphosphate nor inosine triphosphate could be substituted for ATP (6). The need for a coenzyme in the reaction has also been suggested recently (8, 9).

The requirement for choline in the synthesis of acetylcholine is well established (3, 5). The source of the acetyl group is as yet unknown. Acetic acid has no effect on the synthesis of acetylcholine either aerobically or anaerobically (1, 2, 5, 6). Pyruvate was suggested as an aerobic source by Quastel and his associates (2, 3) and by Baer (10) who synthesized acetylcholine non-enzymatically. Acetoacetate was suggested as an anaerobic source by Stedman and Stedman (11, 12). Citrate, glutamate, and cysteine have been shown by Nachmansohn and John to activate the synthesis of acetylcholine (7), while α-keto acids inhibit it, but the mechanisms of the activation and inhibition have not been described.

In this paper data are presented to show that a completely soluble enzyme system which synthesizes acetylcholine can be extracted from mammalian brain preparations. With this enzyme system, the following components are required for the synthesis of acetylcholine: choline, ATP, a substance able to provide *active acetate* which may be either citrate, *cis*-aconitate, or acetoacetate, and a thermostable coenzyme of as yet unknown composition present in boiled yeast or animal tissue extracts.

Preparations—The enzymes employed in this study were prepared from guinea pig, rabbit, and dog brains. The smaller brains yield more active enzymes, but the larger are more convenient. No qualitative differences have been noted. In early studies, brain homogenates similar to those employed by Nachmansohn and Machado (5) were used. These were prepared by homogenizing whole guinea pig brains in 4 volumes of of ice-cold frog-Ringer-phosphate solution in the glass homogenizer of Potter and Elvehjem (13).

In the later series, acetone powders of rabbit or dog brain were used. They were prepared in a manner similar to that described by Nachmansohn and John (7). Extracts were prepared by grinding the powder in a mortar with an appropriate volume of cold calcium-free frog-Ringer-phosphate solution. The suspension was then centrifuged in the cold room at 2500 R.P.M. for 15 to 20 minutes, yielding a cloudy supernatant fluid which was used in most of these experiments.

The substrates employed in these experiments were available commercially or were prepared in this laboratory.

Boiled extracts were prepared from kidney, brain, or brewers' yeast by grinding them in water, and then placing them in a water bath at 80° for 10 minutes. The juice was obtained by filtering or centrifuging the boiled material. The yeast juice was neutralized with sodium hydroxide before use. Because of its ease of preparation it was used in preference to the animal tissue extracts in most of these experiments.

Since previous workers (6, 7) have shown inactivation of the enzyme by oxygen and by low concentrations of copper, and reactivation by cysteine and reduced glutathione, precautions were taken to insure active preparations. The enzyme extracts were dialyzed anaerobically against 200 volumes of 10^{-3} M cysteine in oxygen-free water. The dialysis time varied from 2 to 18 hours as indicated, and was carried out in the cold room in a rocking dialysis unit.

Acetylcholine was assayed with the eserinized frog rectus muscle by a modification of the method of Chang and Gaddum (14) previously described (15). The frog-Ringer's solution contained 0.115 M NaCl, 0.0027 M KCl, 0.00135 M CaCl$_2$, and 0.0025 M NaHCO$_3$. It was eserinized with 3.0 mg. of eserine sulfate per 100 cc. Since the synthesis of acetylcholine requires high concentrations of potassium, and the frog muscle responds to high potassium concentrations with an increased sensitivity to acetylcholine or with contracture, preliminary experiments were performed to determine the highest concentration of potassium which did not interfere with the response to acetylcholine. This was found to be 0.008 M. Precautions were therefore taken in the choice of aliquot to insure that not

more than 0.006 M potassium was present in the final assay solution. With enzyme solutions inactivated by dialysis the degree of synthesis was sometimes so low that aliquots which furnished the maximum tolerated concentrations of potassium contained insufficient acetylcholine to permit a measurable response with the frog muscle. Such preparations are recorded as synthesizing no acetylcholine, although it is possible that some slight synthesis actually occurred.

A calibration curve with acetylcholine bromide (Merck) was established with each muscle before the assays. Points on this curve were rechecked at frequent intervals during the assay of the experimental samples. Results are expressed as micrograms of acetylcholine bromide, since this was the compound used in calibrating the muscle.

Free acetylcholine was determined on untreated aliquots of the synthesizing system. Total acetylcholine was determined by acidifying

TABLE I

Basic Constituents for Anaerobic Synthesis of Acetylcholine

Constituents	Volume	Final concentration
	cc.	*M*
NaF and substrates..........................	0.1	0.02
Eserine sulfate.............................	0.1	0.002
Choline...................................	0.1	0.005
KCl......................................	0.1	0.03
ATP......................................	0.02	0.003
Boiled juice...............................	0.3 (75 mg. per cc.)	
Ca-free Ringer's phosphate to make 3.5 cc.		

the sample to pH 4.0, boiling for 2 minutes, cooling, and then neutralizing before the assay.

All of the experiments reported in this paper were carried out anaerobically in Thunberg tubes which were incubated at 37° for 1 hour. Unless otherwise specified, the total volume was 3.5 cc. and the final concentrations of the various constituents required for the study of the mechanism of synthesis were as given in Table I.

2.0 cc. of the extract of homogenized brain (400 mg. of fresh tissue) and 1.0 cc. of the extract of the acetone powder (50 or 100 mg.) were used in these experiments.

Results

Brain Homogenates—With brain homogenates, and with extracts derived from them, the observations of previous workers (5–7) were confirmed on

several points. These were (1) that K^+ increased the synthesis of acetylcholine in the test system; (2) that Ca^{++} depressed the synthesis; (3) that an optimum concentration of 0.003 M ATP was required; and (4) that the enzyme could be inactivated by dialysis and that part of the activity could be restored by the addition of citrate or $d(-)$-glutamate.

Our observations differed from those of Nachmansohn and John (7) in showing that citrate was more effective than glutamate in restoring the activity of dialyzed preparations (Table II).

Acetone Powders; Solubility of Enzyme Synthesizing Acetylcholine—With brain acetone powder preparations, it is possible to extract with calcium-free frog-Ringer-phosphate solution, an enzyme system which synthesizes acetylcholine in considerable quantities. Such extracts remain turbid

TABLE II

Effect of Citrate and Glutamate on Rate of Synthesis of Acetylcholine

Each tube contained (a) the extract from 400 mg. of fresh brain; (b) eserine, fluoride, choline, KCl, ATP, and acetate in the concentration previously indicated; (c) citrate or *d*-glutamate as indicated.

Experiment No.	Acetylcholine synthesis per gm. fresh tissue			
	Undialyzed extract	Dialyzed extract		
		No substrate	Citrate	Glutamate
	γ	γ	γ	γ
1	29	6.4	24	16.5
2	42	8.6	30	19.5
3	38	7.8	27	20.5
Average...................	36.3	7.6	27	18.8
% of original activity.......		20.9	74.5	51.7

when centrifuged at ordinary speeds. However, centrifugation for 3 hours at 12,000 R.P.M. results in an extract which is completely clear and shows no Tyndall beam. The fact that such a preparation synthesizes acetylcholine at a higher rate than the turbid suspension from which it was prepared proves that the enzyme is in true solution, and that the insoluble tissue particles inhibit synthesis in some unknown manner. The data are summarized in Table III, which also shows that the enzyme system is stable to freezing and thawing and that all of the acetylcholine synthesized is free. In later work, therefore, no attempt was made to measure bound acetylcholine.

The inhibitory effect upon the synthesis of acetylcholine of the insoluble material in the acetone powder may be seen more strikingly in the results

of the following experiment. With an acetone powder prepared from dog brain, the uncentrifuged suspension containing all of the insoluble material synthesized 390 γ per gm. of powder. After centrifugation for 3 hours at 12,000 R.P.M., it synthesized 630 γ per gm. under identical conditions.

Effect of Dialysis—The somewhat cloudy supernatant fluid obtained by centrifuging the extract of acetone powder for 30 minutes at 2500 R.P.M. was used in all the experiments to be described in the following sections. Such preparations are more readily prepared than those involving sustained high speed centrifugation, and, though somewhat less active, give substantially the same results.

When such preparations are dialyzed for 3 hours or more, most of their activity is lost and can be restored by the addition of citrate, ATP, and

TABLE III

Solubility and Stability to Freezing and Thawing of Enzyme System Synthesizing Acetylcholine

Each tube contained (a) extract from 50 mg. of acetone powder; (b) NaF, eserine, choline, KCl, in the concentrations previously tabulated. Free acetylcholine was determined by direct assay; total acetylcholine, after boiling at pH 4.0 for 2 minutes.

Preparation	Acetylcholine synthesis per gm. acetone powder	
	Free	Total
	γ	γ
Cloudy supernatant fluid after 20 min. centrifugation at 2500 R.P.M.	850	850
Clear supernatant fluid after 3 hrs. centrifugation at 12,000 R.P.M.		
With acetate (0.02 M)	900	900
Without acetate	900	900
Cloudy supernatant fluid twice frozen ($-40°$) and thawed	850	

boiled yeast juice. The addition of any single one of the three components, or of the components in pairs, results in substantially reduced activity (Table IV), while the presence of all three components gives synthesis comparable to that of the undialyzed preparation to which only ATP has been added. In similar experiments employing longer periods of dialysis (10 hours), synthesis was eliminated entirely with only one component added and was diminished considerably with two, but under these conditions approximately 50 per cent of the initial activity was lost, even when all three were added. Short dialysis periods were therefore used in order to study more fully the reactions and mechanisms involved. However, even with short dialysis periods it is clear that citrate, ATP, and another impure component containing perhaps several substances and

probably serving as a coenzyme are required for optimum synthesis. Since this work was completed, the existence of activating cofactors for acet- ylcholine synthesis has been reported by Feldberg and Mann (8) and by Lipmann and Kaplan (9).

Need for Citrate—Since citrate is invariably active in the synthesis, other organic acids have been investigated as substitutes for it. Of the large number which were tried in the presence of ATP and boiled yeast juice, *cis*-aconitic and acetoacetic acids have shown activity. It is interest- ing that glutamic acid, which was active with extracts from homogenized brain, had little activity with extracts from acetone brain powder. Keto acids (pyruvic, ketoglutaric, and oxalacetic) were found to be definitely inhibitory, in agreement with the findings of Nachmansohn and John

TABLE IV

Effect of Dialysis on Acetylcholine Synthesis

Dialysis, 3 hours. The enzyme was from an extract containing 100 mg. of acetone powder of dog brain. All tubes contained choline, NaF, eserine, KCl, and sodium acetate at the usual concentrations. The results are given in micrograms of acetyl- choline bromide per gm. of powder per hour.

Experimental conditions	Acetylcholine formation
No additions...	0
Undialyzed enzyme + ATP.....................................	465
Dialyzed + citrate..	100
" + ATP...	90
" + boiled yeast juice.................................	60
" + " " " + ATP........................	175
" + ATP + citrate......................................	200
" + boiled yeast juice + citrate.....................	185
" + " " " + " + ATP.............	435

(7). Glucose, phosphorylated intermediates in glycolysis, amino acids including cysteine, and β-hydroxybutyric acid were without activity (Table V).

The replacement of citrate by *cis*-aconitate and acetoacetate and the failure of the other substrates to result in synthesis suggested that they might function as sources of acetyl groups for the synthesis of acetylcholine. This hypothesis has been tested by larger scale experiments.

Synthesis from Acetoacetic Acid—Two large scale experiments were performed to follow the synthesis of acetylcholine from acetoacetic acid analytically. In these experiments the dialyzed extract from 3 gm. of brain powder was tested. To it were added NaF, eserine, choline, K^+, ATP, and boiled yeast juice to give the same final concentrations

employed in the small scale experiments. The preparation was then made up to 48 cc. with frog-Ringer-phosphate solution and divided into three aliquots of 16.0 cc. each. To the first, 4.0 cc. of the Ringer's solution was added. To the remaining two were added 4.0 cc. of 0.1 M sodium acetoacetate to give a final concentration of 0.02 M. One of these was immediately placed in an ice bath. The other and the control without acetoacetate were incubated anaerobically at 37° for 2 hours. At the

TABLE V

Effect of Substrates on Synthesis of Acetylcholine

The Thunberg tubes contained the extract from 50 mg. of acetone powder of dog brain dialyzed 3 hours. All tubes contained choline, NaF, eserine, KCl, ATP, and boiled yeast juice at the usual concentrations. Substrates, 0.02 M. The results are given in micrograms of acetylcholine bromide synthesized per gm. per hour.

Substrate	Acetylcholine synthesis	Substrate	Acetylcholine synthesis
None	80	Citrate	440
Glycine	90	Hexose diphosphate	80
Acetate	90	d-3-Phosphoglyceraldehyde	80
Cysteine	120	β-Hydroxybutyrate	80
Glutamate	130	Pyruvate	None
Acetoacetate	300	Oxalacetate	"
cis-Aconitate	400	α-Ketoglutarate	"

TABLE VI

Synthesis of Acetylcholine with Acetoacetate As Acetyl Donor

The results are given in micromoles of substance found.

Experimental conditions	Acetoacetic acid added	Acetic acid found	Acetylcholine found
Flask kept at 0°...............................	200	4.2	0
" incubated at 37° for 1 hr.....................	0	4.8	0.53
" " " 37° " 1 "	200	7.5	2.88

end of this period, aliquots were taken for acetylcholine analysis with the frog rectus muscle, and for acetic acid analysis by the method described by Friedemann (16) after refluxing with HgO and double steam distillation. The results cannot be considered entirely satisfactory since the blanks were high and the quantities of volatile acid formed were too small to be titrated with a high degree of accuracy. They show, however, that in the presence of acetoacetic acid quantities of volatile acid sufficient to account for the acetylcholine synthesized are produced (Table VI). It

may be noted that sufficient quantities of volatile acid are initially present in the enzyme and boiled juice to account for more than the quantities of acetylcholine synthesized. The volatile acid is probably acetic acid, whose inactivity in the synthesis has been noted in this paper (Tables III and V) and by previous workers (1, 2, 5, 6).

Citrate in Synthesis of Acetylcholine—The rôle of citrate in the synthesis of acetylcholine has been studied by inhibitors and chemical analyses. As indicated previously, citrate and *cis*-aconitate appear to function interchangeably in the synthesis of acetylcholine, suggesting the presence of aconitase, which has been found to be present in brain (17). In these experiments citrate was used mainly as a substrate, because of its greater availability, but undoubtedly identical results would have been obtained with *cis*-aconitate.

TABLE VII

Effect of Malonate and Semicarbazide upon Synthesis of Acetylcholine

Each tube contained the dialyzed extract from 50 mg. of powder and the usual quantities of NaF, eserine, KCl, ATP, boiled yeast juice, and citrate. 0.02 M malonate and 0.01 M semicarbazide were added as indicated. The results are given in micrograms of acetylcholine bromide per gm. of brain powder per hour.

Experimental conditions	Acetylcholine synthesis
Control..	250
Malonate..	220
Control..	460
Semicarbazide....................................	800
Control..	350
Semicarbazide....................................	500

Effect of Malonate and Semicarbazide—Malonate, an inhibitor of succinate oxidation, was added to prevent the formation of acetate by oxidative steps in the citric acid cycle. In agreement with the results of Feldberg (6), it had no effect upon the synthesis of acetylcholine (Table VII).

Semicarbazide was added to the test system because keto acids inhibit the synthesis of acetylcholine and because the most likely source of acetate from citrate would be the anaerobic breakdown of citrate to yield acetate and oxalacetate. Binding the oxalacetate with semicarbazide might then be expected to increase the rate of acetylcholine synthesis. Table VII shows that semicarbazide increased the rate of synthesis appreciably.

Large Scale Experiments with Citrate—Two large scale experiments have been performed to determine the rôle of the citrate in the synthesis of acetylcholine. For these experiments the dialyzed enzyme from 1 gm.

of acetone powder was brought to a final volume of 50 cc. with the usual concentrations of NaF, eserine, KCl, choline, citrate, ATP, and boiled yeast juice. In addition, semicarbazide was added to a final concentration of 0.01 M. 10 cc. of the contents were immediately stored in the cold room; the remainder was incubated anaerobically at 37° for 1 hour. At the end of the experiment, aliquots were taken from each sample for acetylcholine analysis by the usual method. Other aliquots were precipitated by adding $\frac{1}{5}$ volume of 50 per cent trichloroacetic acid. To these was then added 2,4-dinitrophenylhydrazine in 2 N HCl. After standing for 30 minutes, the quantities of the 2,4-dinitrophenylhydrazone formed were too small for isolation. The keto acid was therefore determined colorimetrically as the 2,4-dinitrophenylhydrazone by the method of Friedemann and Haugen, with ethyl acetate (18). A calibration curve was prepared under identical conditions with known quantities of oxalacetic acid, and the keto acid concentration calculated on the assumption that

TABLE VIII

Synthesis of Acetylcholine with Citrate As Acetyl Donor

The results are given in micromoles per gm.

Substance analyzed	Experiment 1	Experiment 2
Initial keto acid	1.97	1.37
Final keto acid	3.03	2.89
Keto acid formed	1.06	1.52
Initial acetylcholine	0	0
Final acetylcholine	1.66	1.85

it was oxalacetic acid. The results are shown in Table VIII. It may be seen that on a molar basis the quantities of acetylcholine formed agree reasonably well with those of the keto acid formed when the latter is calculated as oxalacetic acid. The correspondence is undoubtedly closer than the data show, since some acetylcholine is initially present in the enzyme preparation, but the quantities are too small to assay with accuracy. Since the anaerobic breakdown of citric acid to acetic acid would theoretically leave oxalacetic acid as a residue, the fact that the quantity of keto acid formed (calculated as oxalacetic acid) agrees with the quantity of acetylcholine formed strongly suggests that the keto acid is indeed oxalacetic acid. Therefore, the rôle of citric acid in the synthesis of acetylcholine appears to involve its anaerobic breakdown to acetate and oxalacetate.

Effect of Acetate—The various workers who have studied the synthesis of acetylcholine have agreed that acetate does not increase the synthesis

and so cannot serve as a source of acetyl groups. Our observations in general are in accord with those of previous workers. However, we have found that, although acetate itself does not increase the synthesis of acetylcholine, and cannot be substituted for citrate, *cis*-aconitate, or acetoacetate, it nevertheless increases the synthesis slightly when added in addition to citrate to the enzyme system. The effect is small or absent in the absence of boiled yeast juice, but is definite when boiled yeast juice, ATP, and citrate are present (Table IX).

Nature of the Coenzymes—No success has yet been attained in substituting known compounds for the boiled yeast juice. Magnesium (100 γ), manganese (100 γ), diphosphopyridine nucleotide (1 mg. of 25 per

TABLE IX

Effect of Acetate on Synthesis of Acetylcholine

Each vessel contained the dialyzed extract from 50 mg. of dog brain and the usual quantities of KCl, NaF, eserine, and choline. ATP, boiled yeast juice, and citrate (0.02 M) were added as indicated. The results are given in micrograms of acetylcholine bromide per gm. per hour.

Experiment No.		Acetylcholine synthesis	
		No acetate	Acetate
1	ATP + citrate	155	200
	" + " + boiled juice	275	435
2	" + "	150	165
	" + " + boiled juice	200	360
3	" + "	180	140
	" + " + boiled juice	245	290

cent DPN), diphosphothiamine (10 γ), and guanine have been tried alone and in combination with negative results.

DISCUSSION

From the work of previous investigators and from the experiments reported in this paper it can be seen that the soluble enzyme system from acetone-dried brain preparations requires at least five components in order to synthesize acetylcholine. These are choline, a suitable substrate, adenosine triphosphate, potassium, and a thermostable component present in brewers' yeast and animal tissues.

The substrates which produced the greatest rates of acetylcholine synthesis were citrate, *cis*-aconitate, and acetoacetate. Evidence has been presented to show that, with acetoacetate as substrate, an increase in volatile acid production, determined chemically, quantitatively parallels

the increase in acetylcholine synthesis, while with citric acid as substrate an increase in keto acid production may be demonstrated along with the synthesis of acetylcholine. It is probable therefore that the substrates active in acetylcholine synthesis are donors of the acetyl groups required in the synthesis. The following reactions have been proposed.

$$\text{Acetoacetate} + H_2O \rightleftharpoons 2 \text{ acetate (active)} \tag{1}$$
$$\text{Citrate} \rightleftharpoons \text{oxalacetate} + \text{.acetate (active)} \tag{2}$$
$$\text{Acetate (active)} + \text{choline} \rightleftharpoons \text{acetylcholine} \tag{3}$$
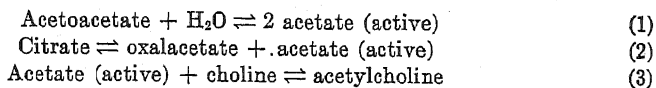
These reactions have not as yet been conclusively proved, since the enzyme system involved, while yielding more than sufficient quantities of acetylcholine for quantitative bioassay, has not yielded sufficient quantities of the other products for isolation and positive identification. Similarly, the quantities of the reacting substrates utilized have been too small to permit their accurate determination. Nevertheless, the reactions proposed form the best working hypothesis for our results and are in accord with the results of other workers.

The formation of acetate from acetoacetate has been shown by Lehninger (19) to take place in muscle, kidney, and bacteria. The reverse reaction, *i.e.* synthesis of acetoacetate from acetate, has also been demonstrated (20). Furthermore, Lipmann (21) has shown that acetoacetate can act in the acetylation of sulfanilamide. The work of these authors is in accord with our conclusion that the condensation of acetate to acetoacetate is reversible, and that acetoacetate may yield acetate groups for a variety of later reactions, of which the acetylation of choline and sulfanilamide is an example.

Neither Nachmansohn and John (7) nor Feldberg and Mann (8), who have reported the enhancement of acetylcholine synthesis by citrate, have offered suggestions for the mechanism of this enhancement. We suggest that citrate acts as an acetyl donor in this enzyme system according to equation (2).

In our experiments acetate cannot be obtained from citrate by oxidative reactions in the system because the experiments are entirely anaerobic. Furthermore, malonate does not inhibit the synthesis. The evidence for the anaerobic breakdown of citrate to yield oxalacetate and acetate is based upon the stimulating effect of citrate upon the synthesis of acetylcholine with the dialyzed enzyme system, augmentation of the stimulation by semicarbazide, and the demonstration of keto acid formation in the course of the reaction. Reactions similar to this have been previously described. The formation of citrate from acetate and oxalacetate has been demonstrated in yeast (22), but has not yet been established in animal

tissues.   Wieland and Rosenthal (23) were unable to find increased syn-
thesis of citric acid by chopped kidney in the presence of acetate and
oxalacetate, although oxalacetate and either pyruvate or acetoacetate
were effective.   The oxidation of pyruvate to acetate has been demon-
strated to occur in kidney (24).   On the other hand, Weinhouse *et al.*
(25) have recently concluded that "citric acid as such is not in the direct
pathway of acetate oxidation in kidney."   Citric acid has been chosen
as one of the members of equation (2) because *cis*-aconitic acid produced
less acetylcholine than did citric acid.

The experiments presented in this paper indicate that soluble enzymes
from brain acetone powder anaerobically split citrate with the formation
of a keto acid and acetate.   The increased formation of acetylcholine
observed on addition of acetate (Table IX) would favor this assumption;
the added acetate would produce a more efficient utilization of the "active"
acetate formed by citrate.

A similar reaction would be responsible for the synthesis of acetylcholine
from *cis*-aconitate, since this is transformed into citrate by aconitase
present in brain (17).   The inhibitory effect of keto acids upon the syn-
thesis of acetylcholine may find an explanation either in the reversal of
reaction (3) towards citrate or by competition with choline for the active
acetate for other condensation reactions.   The synthesis of acetylcholine
by brain homogenates in the presence of glutamate must be due to for-
mation of citric acid, a possibility which was discussed by Adler *et al.*
(26).   The water-soluble extracts from acetone powders used in the ex-
periments presented in this paper apparently contain smaller amounts
of glutamic acid oxidase than the suspensions of homogenized brain used
by Nachmansohn and John (7), hence, the smaller acetylcholine synthesis
with this substrate.

We have postulated the formation of "active" acetate because of the
inability of acetate to enhance the synthesis of acetylcholine in the absence
of citrate or acetoacetate.   This "active" acetate is perhaps a free radical
of very short life, able to produce acetylations or other condensation
reactions.

Whether ATP is required for the formation of "active" acetate from
the more stable precursors, the transformation of the "active" acetate
into acetyl phosphate, which might then be the acetylating agent, or
for the acetylation reaction, as Lipmann (21) postulates, is not yet known.

Similarly, little is known about the rôle or the nature of the thermostable
coenzyme except that it must be a substance of small molecular weight
(less than 10,000), as it freely diffuses through cellophane membranes.
Because the synthesis of acetylcholine by the water-soluble brain extracts

is the result of two different reactions, formation of active acetate and acetylation of choline, it is not yet known whether the coenzyme is necessary for the first or the last reaction.

It must be emphasized that the system obtained from acetone-dried brain may not give a true picture of the synthesis of acetylcholine under physiologic conditions, in which the "active" acetate could be provided by the aerobic oxidation of pyruvate, as shown by Quastel and coworkers (2-4).

SUMMARY

The enzyme system which anaerobically synthesizes acetylcholine is water-soluble and can be extracted from acetone-dried preparations of mammalian brain. At least five components are required for full activity of the enzyme system. These are choline, a suitable substrate, potassium, adenosine triphosphate, and a coenzyme present in boiled aqueous extracts of brewers' yeast or animal tissues.

The suitable substrates which have been found are citrate, cis-aconitate, and acetoacetate.

Evidence has been presented for the view that two different processes occur in the anaerobic synthesis of acetylcholine: the formation of "active" acetate from anaerobic breakdown of citrate or acetoacetate, and the acetylation of choline by the "active" acetate. Whether the coenzyme and adenosine triphosphate are required for the first or the second reaction, or for both, is not yet known.

BIBLIOGRAPHY

1. Feldberg, W., *Physiol. Rev.*, **25**, 596 (1945).
2. Mann, P. J. G., Tennenbaum, M., and Quastel, J. H., *Biochem. J.*, **32**, 243 (1938).
3. Mann, P. J. G., Tennenbaum, M., and Quastel, J. H., *Biochem. J.*, **32**, 822 (1939).
4. Mann, P. J. G., Tennenbaum, M., and Quastel, J. H., *Biochem. J.*, **33**, 1506 (1939).
5. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397 (1943).
6. Feldberg, W., *J. Physiol.*, **103**, 367 (1945).
7. Nachmansohn, D., and John, H. M., *J. Biol. Chem.*, **158**, 157 (1945).
8. Feldberg, W., and Mann, P. J. G., *J. Physiol.*, **104**, 17 P (1945).
9. Lipmann, F., and Kaplan, N. O., *J. Biol. Chem.*, **162**, 743 (1946).
10. Baer, E., *J. Biol. Chem.*, **146**, 391 (1942).
11. Stedman, E., and Stedman, E., *Biochem. J.*, **31**, 817 (1937).
12. Stedman, E., and Stedman, E., *Biochem. J.*, **33**, 811 (1939).
13. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
14. Chang, H. C., and Gaddum, J. H., *J. Physiol.*, **79**, 255 (1933).
15. Tobias, J. M., Lipton, M. A., and Lepinat, A. A., *Proc. Soc. Exp. Biol. and Med.*, **61**, 51 (1946).
16. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
17. Johnsohn, W. A., *Biochem. J.*, **33**, 1046 (1939).

18. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
19. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
20. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157**, 751 (1945).
21. Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).
22. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).
23. Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 24 (1945).
24. Barron, E. S. G., Bartlett, G. R., and Kalnitsky, G., *Federation Proc.*, **5**, 120 (1946).
25. Weinhouse, S., Medes, G., Floyd, N. F., and Noda, L., *J. Biol. Chem.*, **161**, 745 (1945).
26. Adler, E., von Euler, H., Gunther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).

BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

V. THE EFFECT OF FIBRINOGEN ON PROTHROMBIN TIME

By H. F. DEUTSCH and H. W. GERARDE

(*From the Department of Physiological Chemistry, University of Wisconsin, Madison*)

A one stage prothrombin determination which makes use of a constant level of fibrinogen has been reported by Thordarson (1). This assay as used with modifications by Eriksen, Jacobsen, and Plum (2) makes use of a fibrinogen solution containing optimum amounts of thromboplastin and Ca ion. To such a solution is added a constant amount of various dilutions of plasma and the time for clot formation is observed. We have found that the concentration of fibrinogen has a marked effect on the one stage prothrombin assay of Quick (3) when the plasma dilution technique (3, 4) is used. The study of this and other factors influencing this assay is the subject of this report.

EXPERIMENTAL

Plasmas from the various animals were obtained by mixing 9 ml. of blood with 1 ml. of 0.1 M sodium oxalate and centrifuging off the cells. Such a plasma was designated according to the usual terminology as 100 per cent plasma. Rabbit brain thromboplastin prepared according to the method of Quick (5) was used exclusively. 1 ml. of the thromboplastin that was 0.0125 M in Ca ions was added to 0.5 ml. of plasma or a dilution thereof. All reactions were carried out at 25° ($\pm 1°$) and the time for the appearance of a clot or of the formation of fibrils of fibrin was determined visually while the reaction mixture was being vigorously agitated by tilting the reaction tube back and forth.

The various plasma proteins used in this work were separated from plasma by ethanol fractionation (6, 7). Except for a few experiments with human fibrinogen, bovine fibrinogen prepared according to the method of Seegers *et al.* (8) that was from 75 to 85 per cent clottable was used in these studies. The fibrinogen showed no evidence of fibrin formation in the presence of thromboplastin and Ca ions in 10 minutes. All proteins used in this work were dissolved in 0.15 M NaCl and adjusted to pH 7.4.

Results

Early in the course of this work it was recognized that marked differences in the clotting times of dilutions of human, dog, and rabbit plasmas resulted

when saline and fibrinogen solutions were used as the diluents. The presence of the fibrinogen, either bovine or human, not only shortened the clotting times in the higher dilutions of the plasma but made it possible to assay plasma dilutions up to 1 per cent for prothrombin. Typical clotting times of rabbit plasma diluted with saline and 0.3 per cent fibrinogen (clottable) are shown in Fig. 1. The determination of the clotting times of saline dilutions of plasma higher than 1:10 (10 per cent) was often difficult and invariably it was found to be extremely difficult to duplicate values. However, duplicable assays were possible even with the fibrinogen-diluted series at plasma concentrations lower than 1 per cent. A 0.3 per cent solution of fibrinogen was used as the diluent since it represents the approximate level usually found in rabbit and human plasmas.
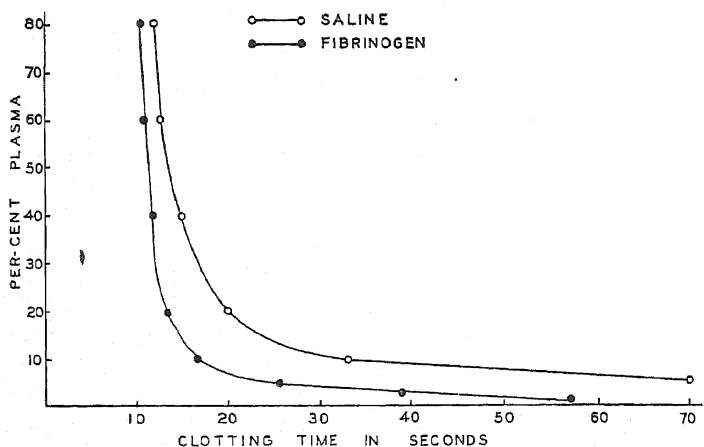
Fig. 1. Effect of diluting rabbit plasma with 0.15 M NaCl and 0.3 per cent bovine fibrinogen in 0.15 M NaCl.

The effect of pH on the clotting times of saline and fibrinogen-diluted plasmas was investigated since in earlier studies (9) it was found that the pH markedly affected the rate and extent of conversion of prothrombin to thrombin, especially in the presence of clotting inhibitors. In this study the pH values were maintained at different levels by use of a buffer that was 0.05 M in both barbiturate and cacodylate. These along with the buffers of the plasma allowed for buffering in the pH range from 6 to 9 and did not remove Ca ion from solution. The components of this buffer did not affect the clotting times of a diluted plasma as compared with the use of saline.

The thromboplastin solutions were prepared in twice the usual concentration and diluted with an equal amount of the above buffer of the desired pH just prior to use. The CaCl$_2$ was dissolved in the buffer solutions. Clotting

times of various plasma dilutions showed minimum values between pH 7 and 8. The higher plasma dilutions, especially the fibrinogen series, showed relatively greater sensitivity to pH changes, but optimum conditions were similar to the saline dilution series. In further studies the pH values of the systems were maintained at pH 7.4.

A wide range of fibrinogen concentrations showed little variation in the clotting times of plasma dilutions. The clotting times of 0.15 and 0.3 per cent fibrinogen concentrations showed minimum values, while higher and lower fibrinogen levels showed slightly lengthened times. For this reason a fibrinogen level of 0.3 per cent was used in future work.
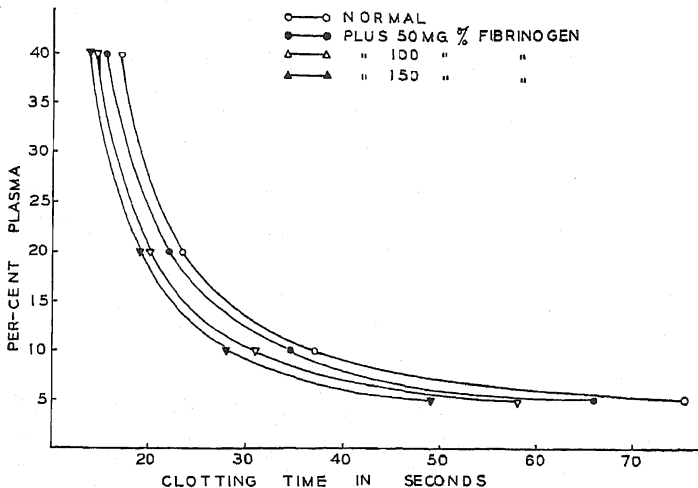
FIG. 2. Effect of fibrinogen increments on the clotting times of rabbit plasmas diluted with 0.15 M NaCl. Fibrinogen added prior to dilution.

Since fibrinogen dilution affected the usual clotting times of diluted plasma, it appeared desirable to study the effect of saline dilution of a plasma that contained variable amounts of fibrinogen but a constant amount of prothrombin. To provide for such a system, several samples of oxalated rabbit plasma were diluted to 80 per cent concentration with fibrinogen solutions sufficient to raise the level of fibrinogen in the original plasmas 50, 100, 150, and 300 mg. per cent. These 80 per cent plasmas were then diluted with saline and the clotting times determined in the usual manner. From Fig. 2 it is obvious that the fibrinogen increments resulted in shorter clotting times. Moreover, the end-points at the higher dilutions of the systems with the higher fibrinogen increments were more easily determined. Thus changes in plasma fibrinogen levels may well affect the clotting times of saline-diluted plasma without influencing the prothrombin time.

72 hours after the feeding of caffeine at the level of 100 mg. per kilo the plasma fibrinogen of rabbits shows a marked increase. The clotting times of saline dilutions of such plasma are shortened, although fibrinogen dilutions of the same plasma show constant or slightly lengthened values, as is seen in Table I. Such data indicate that caffeine does not induce a hyper-prothrombinemia (10) but only an increase in plasma fibrinogen (11). These results parallel the findings shown in Fig. 2 for a system in which direct additions of fibrinogen to plasma resulted in shorter clotting times in the higher saline dilutions of the plasma.

To determine whether other plasma proteins in the concentrations usually found in plasma would affect the clotting times, rabbit plasmas were diluted with 3.5 per cent human serum albumin, 0.5 and 2.0 per cent γ-globulin, and 0.7 per cent β-globulins. These proteins possessed the following purity

TABLE I

Effect of Feeding Caffeine (100 Mg. per Kilo) on Clotting Time

	Clotting time			
	Rabbit A		Rabbit B	
	Before	After	Before	After
Plasma fibrinogen, *mg. %*.........	304	455	306	484
	sec.	*sec.*	*sec.*	*sec.*
10% plasma (saline)	21.1	15.6	16.5	13.5
5% " "	42.7	25.8	33.4	22.7
10% " (fibrinogen)	16.5	15.6	13.4	13.4
5% " "	25.2	23.8	19.6	20.3
1% " "	79.8	82.9	45.7	60.7

as judged by electrophoresis: albumin 99 per cent, γ-globulin 98 per cent, and β-globulin approximately 85 per cent. The β-globulin was analogous to the Fraction III-1 used in our earlier work (9) except that it contained a higher amount of β-globulin. Since this fraction possessed some pro-thrombin activity, it was heated at 55–57° for 1 hour prior to its use in order to inactivate the small amount of prothrombin present. Dilution of plasma with albumin and γ-globulin in the above concentrations did not alter the clotting times as compared with saline solution. The β-globulin, however, had a definite retarding effect, being particularly noticeable at the higher plasma dilutions.

Since de Sütő-Nagy (12) has indicated that clotting inhibitors in certain tissue fractions may be removed by extraction with certain organic solvents, an attempt was made to remove plasma-clotting inhibitors by such ex-traction and thus alter the usual dilution curve clotting times. Portions of

lyophilized rabbit plasmas were extracted with ether, acetone, absolute ethanol, and a 1:4 absolute ethanol-ether mixture. No appreciable differences in the clotting times of reconstituted solutions of the dried extracted and unextracted plasmas were noted.

The assay of prothrombin by the fibrinogen dilution technique described above is analogous in some respects to both the one stage method of Quick and the two stage method of Warner, Brinkhous, and Smith (13). For this reason it appeared desirable to compare the clotting times of fibrinogen-diluted plasma of different species. As would be expected, the prothrombin levels are closer to values reported by Quick (14) for the one stage assay than those reported by Warner, Brinkhous, and Smith (15) for the two stage
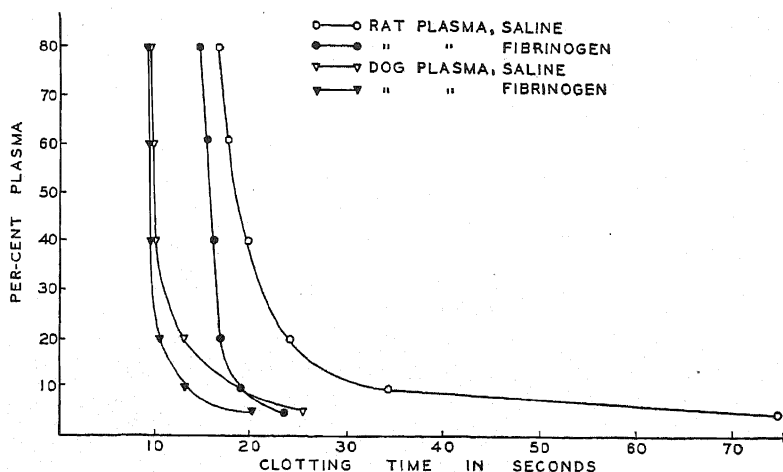
FIG. 3. Comparison of clotting times of plasmas of rat and dog diluted with 0.15 M NaCl and fibrinogen.

assay. Marked deviations in the curves for saline and fibrinogen dilution were experienced for different animals. Fig. 3 shows such a difference for dog and rat plasma.

The work of Quick (16) and of Munroe et al. (17) on prothrombin A and B suggested that the disappearance of the A component might be interpreted as an alteration or lowering of the fibrinogen in the systems studied. However, we were unable to restore the clotting times of aged plasmas to the original value by the addition of fibrinogen. The addition of rabbit plasma, rendered almost free of prothrombin by dicoumarol feeding, to aged rabbit plasma shortened the clotting time of the latter plasma somewhat but did not restore it to its original value. Moreover, addition of fibrinogen to a mixture of 10 per cent aged and 10 per cent dicoumarol plasma did not shorten the clotting time.

The effect of fibrinogen on the one stage assay of prothrombin by the saline dilution curve technique (3, 4) appears to be related to the failure to discern readily the appearance of the clot formed in the higher saline dilutions of plasma owing to the small amounts of fibrinogen present. The addition of fibrinogen circumvents this difficulty. In the usual technique fibrinogen is a limiting factor at the higher plasma dilution and consequently plasma fibrinogen levels will affect the assay. Such a conclusion is obvious from the data in Fig. 2 for rabbit plasma containing variable amounts of fibrinogen but a constant level of prothrombin; progressively shortened clotting times occur at high saline dilutions in the plasma containing the higher amounts of fibrinogen. Experimentally we have been able to duplicate the increased plasma fibrinogen levels and the lowering of the clotting time of saline dilutions of the same plasma of rabbits fed caffeine, as described by Field *et al.* (10, 11). However, in agreement with Quick (18), practically no variation was seen in the higher plasma concentrations. Our most pronounced effect was observed in the 5 per cent saline dilution. The activity of our thromboplastin preparations as reflected by the shorter clotting times of the various dilutions of rabbit plasma studied appears to be greater than those used by Field *et al.* (10). Whether this is a valid criticism of the work of the latter authors, as suggested by Quick (18), is questionable, since we were able to duplicate the essential points of their work, and, moreover, the effect of shortening the clotting time is clearly one of a raised plasma fibrinogen level.

Plasma fibrinogen levels are known to show wide fluctuations (19). Recently in studies on the relation of erythrocyte sedimentation rates and fibrinogen concentrations Rapoport and Guest (20) have indicated that this protein may influence the apparent prothrombin levels. The marked lowering of the plasma fibrinogen levels occasioned by salicylate feeding as noted by these authors may tend to explain the apparent hypoprothrombinemia resulting after feeding of this drug, as reported by Link *et al.* (21) and Field (22). Such a decrease in plasma fibrinogen would tend to lengthen the clotting time of saline dilutions of plasma even though the prothrombin levels remained unchanged. The greater tolerance of dicoumarol by pregnant and lactating rats over normal animals, as reported by Field, Overman, and Baumann (23), may likewise be related to the known rise in plasma fibrinogen occurring during such conditions (19). The hyperprothrombinemia resulting from administration of vitamin K, as reported by Field and Link (24) and by Richards and Shapiro (25), may likewise be a fibrinogen effect. The failure of Quick (26) to substantiate these findings appears to be due to differences in assay method. However, the statement by Richards and Shapiro (25) "that only by the use of diluted plasma is it possible

to detect the hyperprothrombinemia" will not hold if the dilution is carried out with saline as used by these authors. An increased plasma fibrinogen level will affect the assay by making it possible to observe the clot more readily and earlier in the higher saline dilutions. However, a constant fibrinogen level and an increased amount of prothrombin likewise could account for such an effect. We are at present investigating such effects by the simultaneous use of saline and fibrinogen dilution techniques accompanied by fibrinogen assays.

It can readily be seen that by use of fibrinogen solutions the clotting times of plasma are lowered in the higher dilutions and that the assay range can be extended to far more dilute plasmas. Pohle and Stewart (27) have previously made use of a fibrinogen solution in studying the clotting times of various plasma dilutions by the one stage technique. They experienced little difference in the clotting times of 0.5 per cent fibrinogen dilutions of plasma as contrasted with saline dilutions. They did not, however, study plasma diluted beyond 10 per cent.

Quick (28) has commented on the limitations of using the saline dilution technique in prothrombin determinations. The use of fibrinogen rather than $Al(OH)_3$-adsorbed plasma as a plasma diluent, as used by Quick (29, 14), would appear more desirable for several reasons. Use of prothrombin-free plasma as a diluent changes the ratios of all other postulated clotting components to the enzyme being assayed in every dilution. Moreover, $Al(OH)_3$ is not necessarily a specific prothrombin adsorbent and may serve to modify greatly other plasma constituents. The use of a fibrinogen solution circumvents these difficulties.

SUMMARY

The one stage prothrombin assay has been modified by the use of fibrinogen as a diluent of the plasma. This method tends to circumvent the effect of variations in the fibrinogen content of plasma and make this assay more specific for prothrombin. Moreover, the assay range can be extended to much higher dilutions of plasma than is experienced with saline dilutions.

BIBLIOGRAPHY

1. Thordarson, O., Undersogelser over prothrombin hos syge og sunde, Aarhus (1941).
2. Eriksen, F., Jacobsen, E., and Plum, C. M., Acta pharmacol. et toxicol., 1, 379 (1945).
3. Quick, A. J., J. Am. Med. Assn., 110, 1658 (1938).
4. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., J. Biol. Chem., 138, 1 (1941).
5. Quick, A. J., Science, 92, 113 (1940).
6. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., J. Am. Chem. Soc., 68, 459 (1946).

7. Deutsch, H. F., Gosting, L. J., Alberty, R. A., and Williams, J., *J. Biol. Chem.*, **164**, 109 (1946).

8. Seegers, W. H., Nieft, M. L., and Vandenbelt, J. M., *Arch. Biochem.*, **7**, 15 (1945).

9. Deutsch, H. F., *J. Clin. Invest.*, **25**, 37 (1946).

10. Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, **156**, 725 (1944).

11. Field, J. B., Sveinbjornsson, A., and Link, K. P., *J. Biol. Chem.*, **159**, 525 (1945).

12. de Sütö-Nagy, G. J., *J. Biol. Chem.*, **156**, 433 (1944).

13. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, **114**, 667 (1936).

14. Quick, A. J., The hemorrhagic diseases and the physiology of hemostasis, Springfield, 39 (1942).

15. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, **125**, 296 (1939).

16. Quick, A. J., *Am. J. Physiol.*, **140**, 212 (1943).

17. Munroe, F. L., Hart, E. R., Munroe, M. P., and Walkling, A. A., *Am. J. Physiol.*, **145**, 206 (1945).

18. Quick, A. J., *J. Biol. Chem.*, **161**, 33 (1945).

19. Ham, T. H., and Curtis, F. C., *Medicine*, **17**, 413 (1938).

20. Rapoport, S., and Guest, G. M., *Proc. Soc. Exp. Biol. and Med.*, **61**, 43 (1946).

21. Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, **147**, 463 (1943).

22. Field, J. B., *Am. J. Physiol.*, **143**, 238 (1945).

23. Field, J. B., Overman, R. S., and Baumann, C. A., *Am. J. Physiol.*, **137**, 509 (1942).

24. Field, J. B., and Link, K. P., *J. Biol. Chem.*, **156**, 739 (1944).

25. Richards, R. K., and Shapiro, S., *J. Pharmacol. and Exp. Therap.*, **84**, 93 (1945).

26. Quick, A. J., *J. Lab. and Clin. Med.*, **31**, 79 (1946).

27. Pohle, F. J., and Stewart, J. K., *Am. J. Med. Sc.*, **198**, 622 (1939).

28. Quick, A. J., *Am. J. Clin. Path.*, **15**, 560 (1945).

29. Quick, A. J., *J. Immunol.*, **29**, 87 (1935).

LETTERS TO THE EDITORS

TOXICITY OF TYROSINE IN PYRIDOXINE-DEFICIENT RATS
Sirs:

In 1941, Martin *et al.*[1] demonstrated the effect of dopa in increasing blood pressure of dogs rendered hypertensive by the perinephritic technique. Reports were made on the hypertension in rats fed diets containing 5 and 10 per cent *l*-tyrosine[2] and subsequently on the increased toxicity of *l*-tyrosine in the riboflavin-deficient rats.[3]

The involvement of pyridoxine and pyridoxal[4] in tyrosine decarboxylase mechanisms in bacteria suggested the possibility that a pyridoxine deficiency in the rat would decrease the toxicity of *l*-tyrosine. Experiments designed to test this possibility involved the use of 5 per cent tyrosine diets[2] rendered pyridoxine-deficient by omitting this factor from the synthetic diet. Twenty rats were placed on each diet. The pyridoxine-deficient rats receiving 5 per cent *l*-tyrosine at the end of a 5 month test period were all living and had an average weight 20 per cent in excess of the average weight of the surviving 50 per cent of the series of rats receiving pyridoxine and *l*-tyrosine. At 6 months, when the experiment was terminated, 100 per cent of the animals receiving pyridoxine were dead; 100 per cent of the pyridoxine-deficient rats were alive. Results with control animals receiving no tyrosine indicated pyridoxine synthesis in these rats; 1 per cent sulfasuxidine added to the diet caused 100 per cent deaths in 2 months; without the sulfonamide 100 per cent of the animals were alive at 6 months.

The importance of this observation lies in its possible application to certain types of hypertension in the human being.

Research Laboratories   GUSTAV J. MARTIN
  The National Drug Company
  Philadelphia

[1] Martin, G. J., Ichniowski, C. T., Wisansky, W. A., and Ansbacher, S., *Am. J. Physiol.*, **136**, 66 (1942).

[2] Martin, G. J., *Arch. Biochem.*, **1**, 397 (1943).

[3] Martin, G. J., Abstracts, American Chemical Society, 105th meeting, Cleveland (1944).

[4] Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944). Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **48**, 191 (1944); **50**, 95 (1945).

A NEW METABOLITE OF NICOTINAMIDE

Sirs:

N^1-Methylnicotinamide is the major known derivative of nicotinamide excreted in urine; yet only 10 to 30 per cent of ingested nicotinamide can be accounted for in this form. This low excretion, as well as the poor correlation between excretion and the total nicotinamide intake, can most readily be explained by the further metabolism in the body of N^1-methylnicotinamide.[1]

A reaction by which N^1-methylnicotinamide can be further metabolized was found during the search for a physiological function of the quinine-oxidizing enzyme.[2] The only physiological compounds found to be oxidized by this enzyme were those related to nicotinic acid. N^1-Methylnicotinamide was the most rapidly oxidized, and reacted at a rate easily sufficient to account for its disappearance in the body. From the nature of the reaction catalyzed by this enzyme, the product of the oxidation was assumed to be the corresponding pyridone.

This pyridone has now been isolated from large scale oxidation of N^1-methylnicotinamide by the purified quinine-oxidizing enzyme of rabbit liver. Its ultraviolet absorption is intense, with maxima at 260 and 290 mμ. This property allows direct assay of solutions containing as little as 1 γ per ml. The compound can be isolated by adsorption on Lloyd's reagent, followed by extraction into isobutanol.

By the same procedure the compound has also been isolated from human urine in amounts of about 100 mg. per day following daily doses of 600 to 900 mg. of nicotinamide. Its melting point is 212–215°, and the mixed melting point with the enzymically prepared compound, 212–213.5°. The absorption curves of the compounds from both sources are identical.

The analysis is consistent with the formula for one of the pyridones of N^1-methylnicotinamide.

$C_7H_8N_2O_2$.   Calculated.   C 55.24, H 5.31, N 18.42
            Found.   " 55.15, " 5.31, " 18.45

By conversion to the corresponding acid (m.p. 235–237° uncorrected[3]) and the methyl ester (m.p. 137°, uncorrected[4]) and by comparison with the

[1] Perlzweig, W. A., and Huff, J. W., *J. Biol. Chem.*, **161,** 417 (1945). Ellinger, P., and Coulson, R. A., *Biochem. J.*, **38,** 265 (1944). Denko, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Friedemann, T. E., and Youmans, J. B., *Arch. Biochem.*, **10,** 33 (1946).

[2] Knox, W. E., *J. Biol. Chem.*, **163,** 699 (1946).

[3] von Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, **17,** 2384 (1884).

[4] Meyer, H., *Monatsh. Chem.*, **26,** 1311 (1905).

synthetic acid, the metabolite has been identified as the 6-pyridone (N^1-methyl-6-pyridone-3-carboxylamide), the formula of which is given.
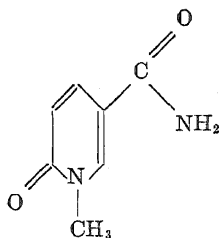
From the amount actually isolated from urine despite losses, it would appear that this pyridone is excreted in an amount at least comparable to that of N^1-methylnicotinamide.

Enzyme Laboratory, Department of Medicine
  College of Physicians and Surgeons
  Columbia University
  New York

W. Eugene Knox
William I. Grossman

STREPTOMYCIN AND DESOXYRIBONUCLEASE IN THE STUDY OF VARIATIONS IN THE PROPERTIES OF A BACTERIAL VIRUS

Sirs:

T_2 bacteriophage, reproduced in *E. coli* B grown in a lactate medium,[1] contained 37 per cent of desoxyribose nucleic acid (DNA) and 3.7 per cent P.[2] Ultraviolet irradiation of T_2 disrupted the limiting membrane of the particle and liberated non-sedimentable DNA into solution.[3] Although most of the DNA is organized within the virus, 30 per cent of the total DNA appears to be organized at the surface of T_2-F.

Streptomycin contains the diguanido base, streptidine. It has been found that streptomycin combines with nucleic acids to produce polymeric

Stability of T_2

Virus preparation	Per cent of initial virus activity				
	0 hr.	1 hr.	2 hrs.	5 hrs.	6 hrs.
T_2-F	100	75	56	28	22
T_2-F after DNase	100	87	79	59	52
T_2-N	100	94	79	68	49

compounds whose size depended on the combining ratios of the bivalent base to multivalent nucleates.[4] At some ratios lattice formation continued until precipitates formed. Similar compounds were produced with T_2-F.

Streptomycin nucleates and complexes of virus and streptomycin were dissociated in M NaCl, as were thymus nucleohistone and nucleoprotamines,[5] which contain many guanido groups.

The high viscosity of T_2 concentrated by differential centrifugation was specifically reduced by desoxyribonuclease (DNase) and not by ribonuclease, trypsin, or lysozyme. DNase did not reduce the activity of the virus. After DNase digestion, 30 per cent of the previously sedimentable DNA of T_2-F was separable from the virus by high speed centrifugation. The DNase-treated virus was not precipitable by streptomycin.

[1] Virus produced by parasitizing *E. coli* in lactate medium (F) or nutrient broth will be termed T_2-F or T_2-N respectively.

[2] Cohen, S. S., and Anderson, T. F., *J. Exp. Med.*, in press.

[3] Anderson, T. F., *J. Cell. and Comp. Physiol.*, **25**, 1 (1945).

[4] I am indebted to Dr. P. György of this University for suggesting this possibility.

[5] Cohen, S. S., *J. Biol. Chem.*, **158**, 255 (1945).

Concentrates of T_2-N had a low viscosity and were not precipitated by streptomycin. The DNase activity of broth lysates containing T_2-N was high. DNase was not found in lactate lysates.

Purified T_2-F was much less stable at 37° in 0.025 M veronal buffer at pH 7.2 than purified T_2-N. Treatment of T_2-F with 0.0001 per cent DNase for 2 hours at pH 7.2 in veronal buffer made T_2-F as stable as T_2-N (see the table).

Thus T_2-F has an outer layer of DNA removable by DNase. On the other hand, T_2-N appeared to have been degraded already by DNase appearing under the conditions of growth in the host in a specific environment. Variations in that environment produced variations in the chemical composition, surface structure, stability, sensitivity to streptomycin, and viscosity of T_2 bacteriophage. The differences in size and stability of T_2 grown in broth and synthetic media described by Hook et al.[6] and Taylor[7] are also probably due to the DNase effects described above. It is not unlikely that the in vitro reactivity of streptomycin with nucleic acid is related to the in vivo activity of this antibiotic.

Children's Hospital of Philadelphia   SEYMOUR S. COHEN
  Department of Pediatrics
  School of Medicine
  University of Pennsylvania
  Philadelphia

[6] Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., J. Biol. Chem., 165, 241 (1946).

[7] Taylor, A. R., J. Biol. Chem., 165, 271 (1946).

BIOLOGICAL PRECURSORS OF URIC ACID CARBON*

Sirs:

The biological precursors of uric acid carbon have been studied by administering compounds labeled with C^{13} to pigeons. Uric acid was isolated from the excreta, purified, and degraded with alkaline MnO_2 to CO_2, urea, and glyoxylic acid. From the work of Fischer and Ach,[1] it would appear that the CO_2 is derived from carbon 6 of uric acid, urea carbon from carbons 2 and 8, and the aldehyde and carboxyl carbons of glyoxylic

Precursor	Rate given	C^{13} concentration, atoms per cent excess						
		Labeled carbons	Uric acid carbon No.					Respiratory CO_2
			2	8	4	5	6	
	mм per hr.							
*CO₂	0.75	8.13	0.00	0.00	0.07†	0.00†	0.25	0.28
CH₃*COOH	1.00	5.82	2.02	2.10	0.07	0.00	0.22	0.26
CH₃*CHOHCOOH	0.50	8.80	0.00	0.00	0.37	0.00	0.26	0.25
*CH₃*CHOHCOOH	0.50	5.40	0.10	0.10	0.07	0.14	0.09	0.11
NH₂CH₂*COOH	0.50	5.20	0.00	0.00	1.16	0.14	0.11	0.13

* Indicates carbon atoms labeled with C^{13}.

† Calculated from isotopic analysis of the glyoxylic acid semicarbazone. The assumption is made that the distribution of isotope in these two positions is identical to the distribution in the experiment with carboxyl acetate.

acid from carbons 4 and 5 respectively. The CO_2 was recovered as $BaCO_3$, urea was converted to CO_2 by urease, and glyoxylic acid isolated as the semicarbazone. This semicarbazone was degraded by acid permanganate into $2CO_2$ and $HCOOH$, the latter coming from the aldehyde carbon of glyoxylic acid. By reaction of another aliquot of uric acid with $KClO_3$, urea containing carbon 8 was obtained. The other product of this reaction, alloxan, was converted by H_2S to insoluble crystalline alloxantin, which was subsequently oxidized by PbO_2 to CO_2 and to urea which contained carbon 2.

The administration of the isotopic compounds listed in the table led to the formation of uric acid containing C^{13} in the positions noted. The C^{13} concentration of respiratory CO_2 is also reported. In a supplementary

[1] Fischer, E., and Ach, F. R., *Ber. chem. Ges.*, **32**, 2745 (1899).

experiment it was found that the carboxyl carbon of acetate was not a direct precursor of urea carbon in the rat.

From the data presented, the following conclusions have been drawn. (1) CO_2 is the precursor of carbon atom 6 of uric acid. Its incorporation into this position is not readily explained by known CO_2 assimilation reactions. (2) The carboxyl carbon of acetate is the precursor of carbons 2 and 8 of uric acid, thus demonstrating a new path of metabolism of acetate. (3) The high concentration of C^{13} in carbon atom 4 after the administration of carboxyl-labeled glycine indicates that glycine or a metabolic derivative is probably the precursor of carbon atom 4 of uric acid. (4) Carbon atom 4 may be derived from the carboxyl group of either lactate or glycine and carbon 5 from the α- (or β-) carbon of lactate. It is thought, therefore, that lactate may be converted to glycine or a metabolic derivative of glycine by reactions comparable to the conversion of serine to glycine.[2] This suggests that carbon 5 of uric acid is derived from the α-carbon of glycine. (5) Glycine and acetate are not interconvertible in the metabolism of the pigeon. (6) The difference in the precursors of urea carbon in the rat and the ureide carbons of uric acid in the pigeon indicates that these two structurally similar groups have different metabolic origins.

Department of Physiological Chemistry   JOHN C. SONNE
  School of Medicine   JOHN M. BUCHANAN
  University of Pennsylvania   ADELAIDE M. DELLUVA
  Philadelphia

[2] Shemin, D., *J. Biol. Chem.*, **162**, 297 (1946).

YEAST MICROBIOLOGICAL METHOD FOR DETERMINATION OF NICOTINIC ACID*

By WILLIAM L. WILLIAMS†

(From the Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland)

The original microbiological method of Snell and Wright (1) for the determination of nicotinic acid has been widely used. Several workers (2–4) have encountered difficulties with this method and have suggested improvements. The improved methods, although largely eliminating the troublesome "drifts" among values calculated at progressively higher assay levels, the variations in the standard curve, high blank values, and unsatisfactory recoveries still require an incubation of from 40 to 72 hours and titration of the acid produced.

The yeast method described in the present communication possesses advantages of rapidity and convenience as compared to the bacterial method. Assays may be obtained in as short a time as 12 hours and the extent of yeast growth is conveniently determined with a photoelectric colorimeter. Furthermore, this method offers an opportunity for checking assay results with a different type of microorganism and may be useful in the differential determination of certain compounds related to nicotinic acid.

The general techniques are essentially those described by Atkin *et al.* (5) and used for the determination of pyridoxine (6) and pantothenic acid (7).

EXPERIMENTAL

Apparatus—The Evelyn photoelectric colorimeter is used with Filter 660 and the test-tubes provided with the instrument. The test-tubes are shaken in a wooden block 18 × 11¾ × 2 inches in which 75 holes ⅞ inch in diameter and 1¾ inches deep have been bored in staggered rows of five. The Fisher-Kahn shaker or the shaker obtainable from the Precision Scientific Company, Chicago, Illinois, for the Kahn test must be reduced in speed by replacing the pulley supplied by a smaller pulley 1⅜ × ½ inch. When 18 mm. test-tubes and the Lumetron instrument previously described are used (5), this reduction in speed is not necessary.

Standard Nicotinic Acid Solution—100 mg. of U. S. P. nicotinic acid reference standard are weighed and diluted to 100 ml. in a volumetric flask. This solution is refrigerated and may be used as long as 4 months. On the day of assay 1 ml. (1 mg.) of this stock solution is diluted to 1000 ml. 5 ml. of this solution are further diluted to 100 ml., giving a solution of 50 millimicrograms per ml.

Culture and Inoculum—Of seventeen yeasts known to require nicotinic acid (8), the response of *Torula cremoris* (American Type Culture Collection No. 2512) was found to be the most satisfactory. The organism is grown on Difco malt agar 24 hours at 37°. This slant is refrigerated

TABLE I

Composition of Basal Medium

Component	Amount per 10 ml. final medium
	mg.
c.p. glucose, anhydrous	500
KH₂PO₄	3
MgSO₄	1
	ml.
Charcoal-treated peptone (Isbell (9))	1 (= 100 mg.)
Potassium citrate buffer (1 liter contains 100 gm. potassium citrate and 20 gm. citric acid)	0.5
	γ
Biotin	0.25
Thiamine hydrochloride	25
Pyridoxine hydrochloride	25
Calcium pantothenate	25
Inositol	250

and used as a stock culture for a period not over a month. 24 hours before an assay series a fresh transfer is made and incubated at 37°. Yeast from this slant is added to 10 ml. of sterile saline in an Evelyn tube until it reads 15 to 25 per cent absorption in the colorimeter. The contents of this tube are then added to 90 ml. of sterile saline and this is used at the rate of 1 ml. per assay tube. The absolute amount of inoculum is not critical but uniformity is essential in each assay series. Amounts of yeast giving values (per cent absorption) from 5 to 50 in the above procedure have been tested with no effect on the assay results. Turbidity values over 20 per cent simply have the effect of adding minute quantities of nicotinic acid to the basal medium.

Basal Medium—The composition of the basal medium is given in Table I. The ingredients can be conveniently combined by preparing three

solutions, (a) charcoal-treated peptone 10 per cent (9), (b) potassium citrate buffer, and (c) vitamins, biotin 1, pyridoxine 100, thiamine hydrochloride 100, calcium pantothenate 100 γ per ml., and inositol 1 mg. per ml.

To prepare, for example, the medium for 100 tubes, 100 ml. of (a), 50 ml. of (b), and 25 ml. of (c) are combined with 50 gm. of glucose, 300 mg. of dihydrogen potassium phosphate, and 100 mg. of magnesium sulfate. This is diluted to 500 ml. (double strength) and dispensed 5 ml. per assay tube.

Besides nicotinic acid, the organism requires only biotin and pantothenate or β-alanine; however, thiamine, inositol, and pyridoxine increase the rate of growth in the early stages. With ammonium sulfate as the nitrogen source and β-alanine instead of calcium pantothenate, the organism requires potassium, magnesium, calcium, sulfur, and phosphorus. With the recommended medium only phosphorus and potassium are required.

The charcoal-treated peptone prepared according to Isbell (9), compared to casein hydrolysate and simpler nitrogen sources, improves the response of the organism to nicotinic acid. Whether this is due to its superiority as a nitrogen source or to unknown growth substances occurring in the peptone is not known.

The contention that the recommended basal medium is complete is supported by the finding that the multiple addition, in excess, of p-aminobenzoic acid, choline, adenine, guanine, uracil, riboflavin, and folic acid and the salts, calcium chloride, ferric chloride, manganese sulfate, copper sulfate, and zinc sulfate, did not affect the standard curve or assays of skim milk powder, dried yeast, and whole wheat flour. Growth on the basal medium plus 2 γ of nicotinic acid per 10 ml. was not improved by the addition of 5 mg. of yeast extract per 10 ml.

Preparation of Samples—As is shown in Table IV, certain naturally occurring methylated derivatives of nicotinic acid are active for *Torula cremoris*. Appropriate treatment as outlined below with 3 N NaOH destroys these substances in pure solutions (Table V) and natural materials (Table II). Trigonelline added to whole wheat is also destroyed by this treatment.

An amount of sample estimated to contain 5 to 10 γ of nicotinic acid is accurately weighed and placed in a 250 ml. beaker. The sample is suspended in the minimum quantity of distilled water, 50 ml. of 3 N NaOH are added, and the solution autoclaved at 15 pounds pressure for 1 hour. After cooling, the pH is adjusted to 5.0 to 5.5 with 3 N H_2SO_4 with the Beckman pH meter or nitrazine paper, and the solution is diluted to 200 ml. If necessary, centrifugation is employed to obtain a clear extract. Frequently materials of high starch content such as wheat flour contain suspended material which cannot be removed by filtering or centrifuging.

In this case the neutralized test solution can be clarified by adding a small amount of taka-diastase (2 to 4 mg.), incubating at 100° for 5 minutes, then centrifuging. Clarase may also be used as described by Atkin *et al.* (6).

<div align="center">

TABLE II

Effect of Sample Treatment on Assay

</div>

Material	Treatment		*Lactobacillus arabinosus* values from literature
	1 N H₂SO₄	3 N NaOH	

Material	1 N H_2SO_4	3 N NaOH	*Lactobacillus arabinosus* values from literature
Substances reported to contain trigonelline or N¹-methylnicotinamide			
	mg. per day	*mg. per day*	*mg. per day*
Urine..	9.8	1.1	1.15 (10)
	γ per gm.	*γ per gm.*	*γ per gm.*
Roasted coffee............................	8900	240	100 (11)
Tomatoes (fresh).........................	37.0	8.8	
Green peas (fresh frozen).................	234	12.5	22 (12)
Substances not known to contain these compounds			
Unenriched flour*.........................	13.2	10.7	11.6 (13)
Enriched flour*...........................	27.2	27.2	25.1 (13)
" bread*...........................	28.4	25.0	26.5 (13)
Whole wheat flour†.......................	50.0	50.5	50.3,‡ 50.3 (4)
Dried yeast†..............................	556	530	591‡
Skim milk powder†........................	8.3		9.0,‡ 8.4 (4)
B complex concentrate No. 4†.............	838	716	803‡
" " " " 5†.............	1320	1170	1340‡
Wilson's liver powder 1:20................	1610	1680	
G. L. F. dog food pellets.................	77.5	46.0	
Hominy grits..............................	17.3	11.5	
Corn germ, raw...........................	199	34.0	34.9 (2)

* Samples used in a collaborative study (13) and obtained from H. K. Steele of The Fleischmann Laboratories, New York.

† Samples used in a collaborative study and obtained from F. M. Strong, University of Wisconsin.

‡ Strong, F. M., personal communication.

Milk and low potency milk products (e.g. skim milk powder) become highly colored by the alkaline treatment, causing turbidity measurements to be in error. As there is no evidence that these materials contain trigonelline or N¹-methylnicotinamide, 1 N H_2SO_4 may be substituted for the 3 N NaOH in the above procedure for this type of material.

Assay Procedure—To each of a series of standard Evelyn test-tubes 5 ml. of basal medium (double strength) are added. The standard nico-

tinic acid solution is added to eight of these test-tubes in the quantities
‘0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 ml., giving respectively 0, 25, 50, 75,
100, 125, 150, and 200 millimicrograms per tube. Typical values for

TABLE III

Nicotinic Acid Assays and Recoveries

Material	Sample per tube	Nicotinic acid found	Nicotinic acid per gm.	Recovery
	mg.	*millimicro-grams*	*γ*	*per cent*
Whole wheat	1	47	47	
	2	88	44	
	3	132	44	
	4	171	43	
	1 + 50 milli-microgramse Nicotinic acid	95		96
	2 + 100 milli-micrograms Nicotinic acid	185		97
Wilson's liver powder 1:20	0.025	44	1760	
	0.05	82	1640	
	0.075	124	1650	
	0.10	167	1670	
	0.025 + 50 milli-micrograms Nicotinic acid	92		96
	0.05 + 100 milli-micrograms Nicotinic acid	181		99
Dried yeast	0.05	26	520	
	0.10	53	530	
	0.15	81	540	
	0.20	106	530	
	0.05 + 50 milli-micrograms Nicotinic acid	75		98
	0.10 + 100 milli-micrograms Nicotinic acid	148		95

growth, obtained from this series and expressed as per cent absorption,
are 11, 20.5, 29, 37, 43.5, 49, 53.5, and 61.5. Four test-tubes are used
for each sample, to which are added 1.0, 2.0, 3.0, and 4.0 ml. of the final
clear sample solution. By the addition of appropriate amounts of distilled
water, the volume of each test-tube is adjusted to 9 ml. The tubes are

plugged with cotton and heated in flowing steam for 10 minutes, cooled, and 1 ml. of inoculum added to each. The assay series is then shaken at any temperature from 30° to 38°. Readings are usually made after 16 and 18 hours; satisfactory results may be obtained, however, as early as 12 hours or as late as 40 hours. By referring the growth attained on the unknown samples to the standard curve, assay values are calculated in the usual manner. Some of the assays obtained are shown in Table II.

TABLE IV

Per Cent Activity of Compounds Related to Nicotinic Acid (Molar Basis)

Compound	Untreated	Treated,* 50 ml. liquid at 120° for ½ hr.	
		1 N H_2SO_4	1 N NaOH
Nicotinic acid	100	97	97.2
Nicotinamide	97.6	98	95
Trigonelline	85	85	76
Nipecotic acid	0	0	0
Pyridine-3-sulfonic acid	0	0	0
Isonicotinic acid	0	0	0
N^1-Methylnicotinamide chloride	0	74	60
Nicotine	0	0	0
Methyl nicotinate	0	0	132
Nicotinonitrile	92	67	106
6-Methylnicotinic acid	0	0	0
Picolinic acid	0	0	0
Coenzyme II (triphosphopyridine nucleotide)	60	121	103
Diethylnicotinamide (coramine)	0	0	121
2-Methylpyridine	0	0	0
3-Methylpyridine	0	0	0
Ethyl nicotinate†	68	74	89
Quinolinic acid†	0	25	0
Nicotinuric " †	0	17	101

* 10 mg. weighed accurately and diluted to 100 ml. 1 ml. (100 γ) treated and diluted as necessary for assay.

† Heated 1 hour as above.

Recoveries of Added Nicotinic Acid—The recovery of the growth factor added to an unknown is considered one test of the validity of a microbiological method. Table III shows the recoveries of nicotinic acid added to whole wheat, Wilson's liver powder 1:20, and dried yeast. Each value is the average of duplicate determinations. The average recovery value of 96.8 per cent is considered satisfactory.

Specificity of Test—In order to gain information on the specificity of the

nicotinic acid molecule for *Torula cremoris*, the activity of a number of related substances was determined as shown in Table IV. To detect any possible inhibitory effects the quantities of the compound tested were superimposed on known amounts of nicotinic acid. No inhibition was

TABLE V

Effect of Acid and Alkali on Nicotinic Acid Activity of Trigonelline and
*N^1-Methylnicotinamide Chloride**

Treatment†	Trigonelline	N^1-Methylnicotinamide chloride
None	81.3	0
H_2O, 1 hr.	91.5	0
1 N H_2SO_4, ½ hr.	85.0	74.0
2 " " 1 "	91.5	91.0
4 " " 1 "	85.4	89.5
1 " NaOH ½ "	56.8	59.6
2 " " 1 "	4.7	6.5
3 " " 1 "	0	0
4 " " 1 "	0	0

* Assuming nicotinic acid to be 100 per cent and comparing on a molar basis.
† 100 γ autoclaved at 120° with 50 ml. of the liquid.

TABLE VI

Assay of Known Mixture of Nicotinic Acid, Trigonelline, and
N^1-Methylnicotinamide Chloride

Compound	Amount found*	Corrected value†
	γ	γ
Nicotinic acid..............................	100	100
Trigonelline................................	96	105
N^1-Methylnicotinamide chloride............	87	96

* Amount present, 100 γ. The experimental values are obtained in terms of nicotinic acid, which serves as the standard. These values have been converted here to the equivalent weights of trigonelline and N^1-methylnicotinamide chloride respectively.

† The transformation by 2 N sulfuric acid of trigonelline and N^1-methylnicotin-amide chloride to nicotinic acid or a compound of nicotinic acid activity is only 91.5 and 91 per cent complete respectively (Table V). The trigonelline value is therefore divided by 0.915 and the N^1-methylnicotinamide value by 0.91 to give the corrected values.

observed. Because the activity of the naturally occurring trigonelline and N^1-methylnicotinamide would seriously limit the use of the method for nicotinic acid assay, inactivation of these compounds was studied with the results shown in Table V.

Assay of Mixtures of Nicotinic Acid, Trigonelline, and N¹-Methylnic-
otinamide—The data of Table V suggest that differential assay of these
three compounds may be possible. To test this a synthetic mixture con-
taining 100 γ each of nicotinic acid, trigonelline, and N¹-methylnicotinamide
chloride was prepared. This was subjected to treatment with (a) 50
ml. of water, (b) 50 ml. of 2 N sulfuric acid, and (c) 50 ml. of 3 N sodium
hydroxide, and autoclaved at 15 pounds for 1 hour. The solutions were
neutralized, diluted, and assayed by the yeast method. The activity of
solutions treated as in (a) should be due to nicotinic acid and trigonelline,
the activity in (b) to all three compounds, and the activity in (c) to nico-
tinic acid alone (Table V). By successive subtractions the activity due
to each of these compounds can be determined. The results of the assay

TABLE VII

24 Hour Excretion of Nicotinic Acid Derivatives by Normal Male Adults

Method	Niacin derivatives*	Trigonelline	N¹-Methyl-nicotin-amide
	mg.	*mg.*	*mg.*
Yeast growth (1 subject)...............................	1.1	7.8	2.57
Chemical (14)† (2 subjects)...........................	1.28	12.7	11.4

* In both cases this refers to both nicotinic acid and nicotinamide.
† The figures in parentheses refer to the literature.

of this known mixture are shown in Table VI. It appears that in pure
solutions the three compounds are readily determined with fair accuracy.

These treatments and the method of calculation were then applied to
urine with the results shown in Table VII. Comparative data obtained
chemically by Perlzweig and Huff (14) are also included.

DISCUSSION

The yeast method described does not appear to offer any basic advantages
for accurate estimation of nicotinic acid over the bacterial methods now
in use; however, the particular adaptability of this method for turbidi-
metric determination of yeast growth renders the method more rapid and
convenient. In the author's experience turbidity measurements of bac-
terial growth are complicated by the appreciable color and the frequent
presence of a precipitate in the bacterial medium. The yeast medium,
on the other hand, is clear and colorless.

The validity of strong alkali treatment of samples is well supported.
Oser *et al.* (15), confirmed by Andrews *et al.* (16) and Krehl and Strong
(17), have demonstrated a bound form of nicotinic acid in natural sub-

stances which is not available to *Lactobacillus arabinosus* unless treated with acid or alkali. Krehl *et al.* (18) have shown that this bound form is active for the dog and chick and recommended autoclaving with alkali or acid to liberate it. A similar situation exists for diethylnicotinamide (coramine). This compound has been shown to be active for dogs and humans (19, 20); however, it is inactive for microorganisms unless treated with alkali (21).

The specificity of the method is supported by the following observations: There is no "drift" in calculated assay values with increasing test levels; no change is observed with changes in conditions, *i.e.* temperature, extending time of test, and size of inoculum; nicotinic acid may be recovered from natural products without appreciable loss; assays are in essential agreement with those obtained with the bacterial method; and the interference of known naturally occurring substances can be eliminated.

The activity of nicotinamide as shown in Table IV does not present a criticism of the method, as the nicotinic acid content of substances is generally accepted to include the free acid and the amide. Of the naturally occurring substances, besides trigonelline and N^1-methylnicotinamide, only those which can be readily hydrolyzed to give nicotinic acid are active.

Because nicotinonitrile, coramine, methyl and ethyl nicotinate, and quinolinic acid do not occur in nature (22), the activity of these compounds is not considered a disadvantage of the method.

It is recognized that the experiments on the assay of mixtures of nicotinic acid, trigonelline, and N^1-methylnicotinamide chloride are preliminary in nature. Further studies on the validity of the procedure and the significance of the low values obtained for trigonelline and N^1-methylnicotinamide in urine are planned. It seems unlikely that other compounds should occur in urine and have the same activity that N^1-methylnicotinamide exhibits toward *Torula cremoris*. This compound, originally inactive, is activated by acid and weak alkali and inactivated by strong alkali. The activity of the closely related betaine, trigonelline, is distinctively different. If future work substantiates the specificity of the procedure for assay of mixtures, the method should prove useful in excretion studies.

SUMMARY

A microbiological method for the determination of nicotinic acid with a yeast, *Torula cremoris*, is presented. The method is rapid, 16 to 18 hours being allowed for growth, and possesses the advantage of ease of estimation of growth response by turbidimetric means. The method satisfies the usual criteria of specificity. The use of the method for the differential assay of nicotinic acid, trigonelline, and N^1-methylnicotinamide in mixtures is indicated.

I wish to thank the following persons for their kind donations of various chemicals: Dr. Daniel Melnick, Food Research Laboratories, Inc., Long Island City, New York, for a sample of N^1-methylnicotinamide chloride; Dr. M. Fine, Hoffmann-La Roche, Inc., Nutley, New Jersey, for the same compound; Dr. B. C. Johnson, University of Illinois, for the trigonelline; Dr. E. T. Tisza, Pyridium Corporation, Yonkers, New York, for pyridine-3-sulfonic acid and nicotinonitrile; Dr. Karl Folkers, Merck and Company, Inc., Rahway, New Jersey, for 6-methylnicotinic acid; Dr. Philip Handler, Duke University, Durham, North Carolina, for coenzyme II and iso-nicotinic acid; and Dr. F. M. Strong, University of Wisconsin, for nico-tinonitrile, methyl nicotinate, and nipecotic acid. Nicotinuric acid was synthesized by James E. Cochran, Ph. M. 3/c, V-6, U. S. N. R., of these laboratories. Finally, I wish to acknowledge the encouragement, interest, and inspiration of Dr. Lawrence Atkin, without which this work would not have been undertaken.

BIBLIOGRAPHY

1. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
2. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 471 (1943).
3. Barton-Wright, E. C., *Biochem. J.*, **38**, 314 (1944).
4. Sarett, H. P., Pederson, R. L., and Cheldelin, V. H., *Arch. Biochem.*, **7**, 77 (1945).
5. Atkin, L., Schultz, A. S., and Frey, C. N., *Arch. Biochem.*, **1**, 9 (1942).
6. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
7. Atkin, L., Williams, W. L., Schultz, A. S., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **16**, 67 (1944).
8. Rogosa, M., *J. Bact.*, **46**, 435 (1943).
9. Isbell, H., *Science*, **102**, 671 (1945).
10. Johnson, B. C., Hamilton, T. S., and Mitchell, H. H., *J. Biol. Chem.*, **159**, 231 (1945).
11. Teply, L. J., Krehl, W. A., and Elvehjem, C. A., *Arch. Biochem.*, **6**, 139 (1945).
12. Cheldelin, V. H., and Williams, R. R., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 671 (1942).
13. Steele, H. K., *Cereal Chem.*, **22**, 448 (1945).
14. Perlzweig, W. A., and Huff, J. W., *J. Biol. Chem.*, **161**, 417 (1945).
15. Oser, B. L., Melnick, D., and Siegel, L., Abstracts Division of Biological Chemistry, American Chemical Society, Atlantic City, 31 (1941).
16. Andrews, J. S., Boyd, H. M., and Gortner, W. A., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 663 (1942).
17. Krehl, W. A., and Strong, F. M., *J. Biol. Chem.*, **156**, 1 (1944).
18. Krehl, W. A., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, **156**, 13 (1944).
19. Woolley, D. W., Strong, F. M., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, **124**, 715 (1938).
20. Spies, T. D., Bean, W. B., and Stone, R. E., *J. Am. Med. Assn.*, **111**, 584 (1938).
21. Teply, L. J., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **55**, 72 (1944).
22. Elvehjem, C. A., and Teply, L. J., *Chem. Rev.*, **33**, 185 (1943).

BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

IV. PREVENTION OF PANTOTHENIC ACID SYNTHESIS BY CYSTEIC ACID*

By JOANNE MACOW RAVEL AND WILLIAM SHIVE

(From the Clayton Biochemical Institute and the Department of Chemistry, The University of Texas, Austin)

The study of the metabolism of cells by a method termed *inhibition analysis* (1–3) led to the initial proposal that the antibacterial index (4) is a function of the inhibited enzyme system which becomes the limiting factor for growth of the organism. It was further proposed that if the product of this enzyme system is supplied to the organism the analogue either becomes ineffective as a growth inhibitor or at higher concentrations affects another enzyme system. In the latter case a higher antibacterial index, corresponding to this second enzyme system, is obtained. Precursors of the metabolite may be effective in preventing inhibition of growth by the analogue over a range of concentrations, depending upon the effectiveness with which they are transformed into the metabolite.

In the present investigation paralleling recent work on the inhibition of growth of *Escherichia coli* with hydroxyaspartic acid (1), cysteic acid was found to inhibit growth of that organism but only in the absence of β-alanine or pantothenic acid. The inhibition was competitively prevented by aspartic acid; hence, because of the structural similarity of cysteic acid and aspartic acid, it appears that the enzyme system converting aspartic acid to β-alanine was blocked by the inhibitor. An unusual result was obtained with glutamic acid which was found in many tests to be more effective than aspartic acid in preventing the toxicity of cysteic acid. In order to investigate further what appears to be greater activity of a precursor with respect to the metabolite, tests determining the comparative effects of aspartic acid, glutamic acid, α-ketoglutaric acid, and mixtures of α-ketoglutaric acid and aspartic acid were carried out simultaneously.

Cysteic acid also inhibits the growth of *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5, and *Leuconostoc mesenteroides* P-60, and in all cases the inhibition is reversed competitively by aspartic acid but not by glutamic

---

acid. Details of these experiments and their implications are presented below.

Materials—α-Ketoglutaric acid was prepared by a combination of the methods of Blaise and Gault (5) and Gabriel (6).

TABLE I

Effects of Aspartic Acid, β-Alanine, and Pantothenic Acid on Toxicity of Cysteic Acid for Escherichia coli

Incubated 16 hours at 37–38°.

	l-Cysteic acid	Reversing agent	Galvanometer readings*
	γ *per 10 cc.*	γ *per 10 cc.*	
l-Aspartic acid	0	0	52.0
	100	0	53.5
	300	0	5.5
	0	30	50.0
	300	30	52.0
	1,000	30	2.5
	0	100	54.0
	1,000	100	51.0
	3,000	100	2.0
	0	300	54.0
	3,000	300	49.0
	10,000	300	1.0
	30,000	1000	1.0
	30,000	3000	2.0
Antibacterial index, 30 *ca.*			
Pantothenic acid	0	1.0	55.0
	10,000	1.0	53.0
	30,000	1.0	14.0
	100,000	1.0	2.0
β-Alanine	0	10	53.0
	10,000	10	53.0
	30,000	10	22.0
	100,000	10	6.0
Antibacterial index, >3000 in presence of β-alanine or pantothenic acid			

* A measure of culture turbidity; distilled water reads 0, an opaque object 100.

dl-"*para*"-Hydroxyaspartic acid was prepared by the method of Dakin (7). Only this isomer was used in the tests described in this investigation.

l-Cysteic acid monohydrate was prepared from *l*-cystine by the method described by Clarke (8).

Testing Methods—Tests with *Escherichia coli* were performed as previously described (1). The casein hydrolyzed with trypsin was omitted from the basal medium. For lactic acid bacteria, a previously described procedure was used (9) with a basal medium containing no aspartic acid. Time and temperature of incubation are given in Tables I to IV.

Results—The results as shown in Table I indicate that cysteic acid is toxic to *Escherichia coli* and that the toxicity is prevented competitively by aspartic acid, the antibacterial index being approximately 30. The antibacterial index varied from one test to another but was usually in the range of 30 to 100. However, no inhibition of growth was obtained in a medium containing 1 γ of pantothenic acid or 10 γ of β-alanine until irreversible toxicity with respect to aspartic acid, pantothenic acid, or β-alanine was attained at levels of cysteic acid varying from 30 to 300 mg. per 10 cc.

The inhibition of growth of *Lactobacillus casei* and *Lactobacillus arabinosus* 17-5 by cysteic acid is shown in Table II. The inhibition is prevented competitively in both cases by aspartic acid. The antibacterial index is approximately 300 with either organism. Similar tests with *Leuconostoc mesenteroides* P-60 gave analogous results with an antibacterial index of about 1000. β-Alanine had no effect on the toxicity of cysteic acid for *Lactobacillus arabinosus*, the only species of lactic acid bacteria so tested.

The comparative effects of aspartic acid, glutamic acid, α-ketoglutaric acid, and a mixture of aspartic acid and α-ketoglutaric acid on the toxicity of cysteic acid and hydroxyaspartic acid for *Escherichia coli* are shown in Tables III and IV. In order to standardize the results, simultaneous tests inoculated from the same culture of the organism were repeated several times. Some generalizations can be made concerning the results of these tests. In regard to inhibition of growth with cysteic acid, the following comparisons can be made with regard to the effectiveness of these materials in reversing the toxicity: (1) Aspartic acid prevented the inhibition competitively, the antibacterial index being approximately 30 and sometimes near 100. (2) Glutamic acid was about 3 times as effective as aspartic acid in preventing the inhibition, but often showed some decrease in activity at higher concentrations, 300 γ per 10 cc. (3) α-Ketoglutaric acid was approximately as effective as aspartic acid in reversing the inhibition; however, at concentrations of 100 to 300 γ per 10 cc. the keto acid appeared to become progressively less effective. (4) Mixtures of α-ketoglutaric acid and aspartic acid were equally, if not more, effective than amounts of glutamic acid from which such mixtures could be derived by transamination. Regarding inhibition of growth of *Escherichia coli* with hydroxyaspartic acid, the following generalizations

can be made: (1) Aspartic acid reversed the inhibition competitively, the antibacterial index being 10 to 30.   (2) Glutamic acid was approximately as effective as aspartic acid, except at higher concentrations,

TABLE II

Growth Inhibition by Cysteic Acid and Its Reversal by Aspartic Acid

l-Cysteic acid	*l*-Aspartic acid	Galvanometer readings	
		*Lactobacillus casei**	*Lactobacillus arabinosus*†
γ *per 5 cc.*	γ *per 5 cc.*		
0	0	13.6	32.0
0	10	37.5	
100	10	20.0	
300	10	18.0	
1,000	10	15.2	
3,000	10	5.0	
0	30	56.2	52.0
300	30	22.8	37.0
1,000	30	17.0	26.2
3,000	30	6.0	9.5
10,000	30	4.0	5.0
0	100	60.0	57.0
1,000	100	47.0	59.0
3,000	100	29.8	40.6
10,000	100	19.0	16.0
30,000	100	9.0	8.2
0	300	61.0	69.7
3,000	300	59.0	67.0
10,000	300	62.0	47.0
30,000	300	32.0	26.0
100,000	300	14.5	11.2
300,000	300	8.0	6.0
0	600	62.2	76.0
6,000	600	62.2	70.2
20,000	600	60.5	57.0
60,000	600	31.5	29.5
200,000	600	7.6	6.2
100,000	10,000	36.5	7.2
100,000	30,000	47.5	7.0
Antibacterial index.....	....	300 *Ca.*	300 *Ca.*

* Incubated 3 days at 37–38°.
† Incubated 18 hours at 30°.

100 to 300 γ per 10 cc., at which the relative activity of glutamic acid steadily decreased with increases in concentration.   (3) α-Ketoglutaric acid was approximately as active as aspartic acid only at very low con-

TABLE III

Comparative Effects of Aspartic Acid, Glutamic Acid, and α-Ketoglutaric Acid on Toxicity of Cysteic Acid for Escherichia coli

Incubated 16 hours at 37–38°.

l-Cysteic acid	Galvanometer readings			
	l-Aspartic acid	*l*-Glutamic acid	α-Ketoglutaric acid*	*l*-Aspartic acid and α-ketoglutaric acid*
0 γ per 10 cc.				
γ per 10 cc.				
0	44.0			
30	46.0			
100	7.0			
300	3.0			
10 γ per 10 cc.†				
0	45.0	45.0	44.0	47.0
100	42.0		43.0	
300	6.0	40.0	10.0	37.0
1,000	3.0	19.0	6.0	8.0
3,000		9.0		3.0
10,000		3.0		2.0
30 γ per 10 cc.†				
0	44.0	45.5	44.0	44.0
300	38.0	42.0	41.5	41.0
1,000	7.5	26.5	6.0	41.0
3,000	4.2	13.0	5.0	14.5
10,000		3.0		4.5
100 γ per 10 cc.†				
0	44.5	46.0	44.0	47.0
1,000	42.0	44.8	41.0	39.0
3,000	6.0	42.5	18.0	40.0
10,000	2.0	7.0	4.0	14.0
30,000		3.0		5.0
300 γ per 10 cc.†				
0	45.0	49.0	47.0	45.0
3,000	44.2	45.0	23.0	44.8
10,000	3.5	31.0	4.0	43.2
30,000		4.0		4.8

Antibacterial index, 30 *Ca*.

* Autoclaved separately and added aseptically to the sterilized medium.

† For the mixture of *l*-aspartic and α-ketoglutaric acids, the indicated weight of each was added.

TABLE IV

Comparative Effects of Aspartic Acid, Glutamic Acid, and α-Ketoglutaric Acid on Toxicity of Hydroxyaspartic Acid for Escherichia coli

Incubated 16 hours at 37–38°.

dl-para-Hydroxyaspartic acid	Galvanometer readings			
	l-Aspartic acid	l-Glutamic acid	α-Ketoglutaric acid*	l-Aspartic acid and α-ketoglutaric acid*
0 γ per 10 cc.				
γ per 10 cc.				
0	43.5			
30	42.0			
100	9.8			
300	1.5			
10 γ per 10 cc.†				
0	44.0	44.0	44.3	44.0
30	43.0	45.0	44.0	45.0
100	35.0	42.4	25.5	42.0
300	6.0	5.0	3.2	11.0
1000	2.0	1.2	2.0	2.0
30 γ per 10 cc.†				
0	46.0	47.8	48.0	46.0
100	42.5	41.0	42.0	43.0
300	15.0	10.0	8.0	21.0
1000	3.0	1.5	2.0	9.0
3000	1.5	1.5	2.2	2.7
100 γ per 10 cc.†				
0	44.0	45.0	47.0	47.5
100	43.0	43.5	44.0	44.0
300	27.0	31.0	14.5	31.0
1000	13.0	2.0	2.0	19.0
3000	3.7	1.5	2.0	12.0
300 γ per 10 cc.†				
0	45.0	47.7	47.0	47.0
100	43.5	47.5	44.0	43.5
300	40.0	45.0	18.0	42.0
1000	24.0	2.0	2.0	30.5
3000	19.0	1.5	3.0	29.0

Antibacterial index, 10–30

* Autoclaved separately and added aseptically to the medium.
† For the mixture of l-aspartic and α-ketoglutaric acids, the indicated weight of each was added.

centrations, 10 γ per 10 cc., and further increases in the concentration of the keto acid had little effect on the toxicity of hydroxyaspartic acid. (4) Mixtures of α-ketoglutaric acid and aspartic acid were little if any more active than the amount of aspartic acid in the mixture.

Separate experiments indicated that several other metabolites are somewhat effective in reversing the toxicity of cysteic acid for *Escherichia coli*. Among these were thiamine, leucine, asparagine, arginine, proline, hydroxyproline, isoleucine, and valine.

DISCUSSION

The competitive cysteic acid-aspartic acid growth inhibition obtained with *Escherichia coli* appears to be a function of the enzyme system which decarboxylates aspartic acid to β-alanine. As no other enzyme system utilizing aspartic acid is blocked by cysteic acid, the addition of β-alanine or pantothenic acid to the medium supplies the limiting factor for growth; hence, cysteic acid is then no longer toxic at any level up to 30 mg. per 10 cc., at which level it is irreversibly toxic with respect to aspartic acid, β-alanine, or pantothenic acid. In other words, cysteic acid prevents only one necessary reaction of aspartic acid, its decarboxylation to β-alanine, in *Escherichia coli*. It has not been determined whether cysteic acid is decarboxylated to taurine by the enzyme and merely occupies the enzyme during the reaction or whether cysteic acid forms a complex with the enzyme which does not react further.

The inhibition of growth of *Lactobacillus casei*, *Lactobacillus arabinosus*, and *Leuconostoc mesenteroides* with cysteic acid is competitive in nature; that is, aspartic acid and cysteic acid appear to compete for a common enzyme which performs some function of aspartic acid. This function is not β-alanine synthesis in the case of *Lactobacillus arabinosus*.

The enhanced effect of glutamic acid over aspartic acid in reversing cysteic acid toxicity for *Escherichia coli* is indeed an unusual effect, that of a precursor of a metabolite being more active than the metabolite in reversing an inhibition. Two explanations for this effect have been considered. The first is that α-ketoglutaric acid is converted into glutamic acid by an enzyme system of which the keto acid is the limiting factor. As *Escherichia coli* is known to contain transaminase (10), the conversion of the glutamic acid to aspartic acid and more α-ketoglutaric acid would take place rapidly. The α-ketoglutaric acid could then be used again, the limiting factors for this cycle being the rates of the reactions and the utilization of glutamic acid and α-ketoglutaric acid for other purposes in the organism. By such a process, glutamic acid could show a greater activity than aspartic acid. The second explanation is that α-ketoglutaric acid or some closely related compound may increase the enzyme concentration of the system which decarboxylates aspartic acid.

A study of the comparative effects of aspartic acid, glutamic acid, and α-ketoglutaric acid on the toxicity of cysteic acid and hydroxyaspartic acid for *Escherichia coli* was made as indicated in Tables III and IV in order to determine which, if either, of these two explanations might be correct. A mixture of α-ketoglutaric acid and aspartic acid is equally, if not more, effective than glutamic acid in preventing the toxicity of cysteic acid. Hence, an initial transamination reaction which is known to be rapid for enzyme preparations from *Escherichia coli* (10) is indicated. The effect of glutamic acid, α-ketoglutaric acid, and a mixture of α-ketoglutaric acid and aspartic acid on the toxicity of hydroxyaspartic acid shows that the enhanced effect of glutamic acid over aspartic acid does not exist with this inhibitor. If an increased production of enzyme had resulted, the effect of increased activity of the glutamic acid should be common to both inhibitors since they apparently prevent the functioning of the same enzyme under the testing conditions (1). It might be expected that precursors of aspartic acid should act similarly in preventing the toxicity of each inhibitor. However, the relatively high activity of α-ketoglutaric acid in reversing the toxicity of cysteic acid and the low activity of that keto acid in reversing the toxicity of hydroxyaspartic acid appear to result from the variation in the ability of the organism to transform α-ketoglutaric acid into glutamic acid in the presence of the inhibitors. Such a variation in activity could be explained either by hydroxyaspartic acid directly or indirectly inhibiting this transformation or by cysteic acid aiding the conversion. Cysteic acid and α-ketoglutaric acid have been shown to undergo transamination in the presence of some enzyme preparations (11). Further study is being given this aspect of the problem.

SUMMARY

The toxicity of cysteic acid for *Lactobacillus arabinosus*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, and *Escherichia coli* is prevented competitively by aspartic acid, the antibacterial indices being approximately 300, 300, 1000, and 30, respectively.

Cysteic acid prevents the synthesis of pantothenic acid in *Escherichia coli* by blocking an enzyme of the system which decarboxylates aspartic acid to form β-alanine. Either β-alanine or pantothenic acid completely prevents the toxicity of cysteic acid up to levels of 30 mg. of inhibitor per 10 cc. At this level, cysteic acid is irreversibly toxic with respect to aspartic acid, β-alanine, or pantothenic acid.

Glutamic acid is about 3 times as effective as aspartic acid in preventing

the toxicity of cysteic acid for *Escherichia coli.* A study of the comparative effects of aspartic acid, glutamic acid, α-ketoglutaric acid, and mixtures of α-ketoglutaric acid and aspartic acid on the toxicity of cysteic acid and hydroxyaspartic acid is reported. Some explanations of the enhanced effect of glutamic acid over aspartic acid on the toxicity of cysteic acid are discussed.

BIBLIOGRAPHY

1. Shive, W., and Macow, J., *J. Biol. Chem.*, **162,** 451 (1946).
2. Shive, W., and Roberts, E. C., *J. Biol. Chem.*, **162,** 463 (1946).
3. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **164,** 53 (1946).
4. McIlwain, H., *Brit. J. Exp. Path.*, **23,** 95 (1942).
5. Blaise, E. E., and Gault, H., *Compt. rend. Acad.*, **147,** 198 (1908).
6. Gabriel, S., *Ber. chem. Ges.*, **42,** 655 (1909).
7. Dakin, H. D., *J. Biol. Chem.*, **48,** 273 (1921).
8. Clarke, H. T., in Organic syntheses, New York, **20,** 23 (1939).
9. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152,** 83 (1944).
10. Lichstein, H. C., and Cohen, P. P., *J. Biol. Chem.*, **157,** 85 (1945).
11. Cohen, P. P., *J. Biol. Chem.*, **136,** 565 (1940).

THE EFFECT OF DIETARY PROTEIN UPON AMINO ACID EXCRETION BY RATS AND MICE*

BY H. E. SAUBERLICH AND C. A. BAUMANN

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

Recent progress in microbiological determinations of individual amino acids (1) suggests their application to problems in animal metabolism. Previous communications from this laboratory dealt with tryptophane in blood and urine (2, 3). The present study involves sixteen amino acids in the urine of rats and mice fed diets containing 10 or 50 per cent of casein. For this study existing procedures for certain of the amino acids were used directly, or they were adapted to other acids, *e.g.*, to cystine, proline, tyrosine, and aspartic acid; standard organisms and media were used throughout. Parallel chemical measurements were made of the α-amino nitrogen in the same specimens of urine.

Methods

Care of Animals—Groups of weanling Sprague-Dawley rats were kept singly in screen bottom cages. Albino mice were kept in groups of three to five in similar cages. Food and water were given *ad libitum*, and the animals were weighed at weekly intervals. The diets contained 10 or 50 per cent of purified casein (4), 4 per cent of salts (5), 5 per cent of corn oil (Mazola), a synthetic vitamin mixture (3), and glucose to 100 per cent. 1 part of halibut liver oil was added to 1000 parts of the corn oil used. The animals were maintained on these diets for at least 2 months before the urine was collected. At this time the rats weighed approximately 200 gm. and the mice 25 gm.

Individual rats or groups of three to five mice were placed in metabolism cages fitted with an outside feeder. The urine was collected under a small amount of toluene, and after 24 hours the funnels were rinsed with a minimum amount of distilled water, the urine was removed, and the collection was continued for 6 days. Thus four pooled samples of urine were obtained, two equivalent to 30 mouse days each from mice on 10 or 50

per cent of casein respectively, and two others equivalent to 48 rat days each, from rats on the two levels of protein. The samples were preserved at 0° under toluene.

In addition to the analyses of the pooled samples many determinations were also made on 24 hour specimens of urine from individual rats or groups of three mice each. The variations within groups for the percentage of any ingested amino acid excreted on a given diet were often wide, *e.g.*, from 0.4 to 1.6 per cent of leucine daily per rat on 10 per cent of casein. The average of six determinations, however, was 1.0 as compared to 1.17 on a pooled sample from rats on the same diet. For all other amino acids for which individual analyses were run, satisfactory agreement was also obtained between the averages of individual analyses and the results obtained on the pooled samples of urine.

Determination of Amino Acids—The methods used are essentially those reported previously (6–8). *Lactobacillus arabinosus, Streptococcus faecalis,* and *Leuconostoc mesenteroides* were grown on appropriate media (Table I) from which the amino acid being determined was omitted. All cultures were incubated for 72 hours at 37° and the acidity produced was measured by titration. Medium I was ordinarily used for *Lactobacillus arabinosus* (6), Medium II (7) for *S. faecalis*, and Medium III (8) for *Leuconostoc mesenteroides*. The latter medium could also be used for *Lactobacillus arabinosus* in the determination of certain of the amino acids (Table II). Media IV and V were used with either *Lactobacillus arabinosus* or *Leuconostoc mesenteroides*, and with suitable changes in the buffers, as in Medium II, these media were also found to be suitable for acid production by *S. faecalis*.

In contrast to Media I, II, and III, in which the amino acids were supplied as such, most of the amino acids in Media IV and V were present in the form of casein which has been hydrolyzed with HCl. Medium IV has been used previously in the determination of tryptophane (3, 9). Medium V contained a similar hydrolysate which, however, was prepared from casein that had previously been oxidized with hydrogen peroxide in formic acid solution (10). This procedure largely destroys cystine, methionine, and tryptophane (10, 11). The hydrolysate from such casein proved to be suitable for the determination of each of these amino acids, provided the others were added to the medium; in addition, Medium V also contained added serine, proline, tyrosine, lysine, and glycine. The crude Medium V is not only less expensive than the synthetic Media I to III, but it has yielded more consistent results for the determination of cystine and methionine than the other media attempted by us. In principle, it should, of course, be possible to determine these amino acids with synthetic media, and methionine, for example, has already been so determined (12–15). However, commercially available amino acids apparently are

TABLE I

Media for Microbiological Assays for Amino Acids (All Made Up to 500 Ml.) *

	Medium I	Medium II	Medium III	Medium IV	Medium V
	mg.	mg.	mg.	mg.	mg.
Amino acids					
dl-Leucine	200	200	200		
dl-Isoleucine	200	200	200		
dl-Valine	200	200	200		
l(−)-Cystine	100	200	200	100	100
dl-Methionine	100	100	200		200
dl-Tryptophane	50	100	100	50	100
l(−)-Tyrosine	50	100	100		100
dl-Phenylalanine	100	100	100		
l(+)-Glutamic acid	400	400	400		
dl-Threonine	200	200	200		
dl-α-Alanine	200	100	200		
l-Asparagine	200	200	200		
l(+)-Lysine hydrochloride·H_2O	200	200	200		100
l(+)-Arginine hydrochloride	50	50	100		
l(+)-Histidine hydrochloride·H_2O	50	50	100		
dl-Serine	50	50	200		100
Glycine	20	20	100		100
l(−)-Proline	50	50	50		50
Hydrolyzed oxidized casein					5,000
" regular "				5,000	
Glucose	20,000	20,000	20,000	20,000	20,000
Sodium acetate	20,000		20,000	20,000	20,000
" citrate·H_2O		25,000			
Salts A					
KH_2PO_4	500		500	500	500
K_2HPO_4	500	5,000	500	500	500
Salts B					
$MgSO_4·7H_2O$	200	200	200	200	200
$FeSO_4·7H_2O$	10	10	10	10	10
$MnSO_4·4H_2O$	10	10	10	10	10
NaCl	10	10	10	10	10
Purines and pyrimidines					
Adenine sulfate·$2H_2O$	10	10	10	10	10
Guanine·HCl·$2H_2O$	10	10	10	10	10
Uracil	10	10	10	10	10
Xanthine	10	10	10	10	10
Vitamins					
Thiamine hydrochloride	0.5	0.5	0.5	0.5	0.5
Pyridoxine hydrochloride	1.0	1.0	1.0	1.0	1.0

TABLE I—*Concluded*

	Medium I	Medium II	Medium III	Medium IV	Medium V
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Vitamins—*Continued*					
dl-Calcium pantothenate..	0.5	0.5	0.5	0.5	0.5
Riboflavin...............	0.5	0.5	0.5	0.5	0.5
Nicotinic acid............	1.0	1.0	1.0	1.0	1.0
p-Aminobenzoic acid......	0.1	0.1	0.1	0.1	0.1
Biotin..................	0.001	0.001	0.001	0.001	0.001
Folic acid...............	0.010	0.010	0.010	0.010	0.010

* In all determinations 1 ml. of medium was added to 1 ml. of liquid containing the aliquot of urine being assayed.

not always free from cystine and methionine (15), and synthetic media prepared from certain lots of commercial acids have yielded abnormally high blanks when cystine or methionine was omitted. Furthermore, irregular responses have been observed when graded amounts of cystine were added to certain synthetic media; on the other hand, uniform responses to either amino acid were observed when they were added to the cruder Medium V.

Standard methods were employed for growing the culture organisms and for inoculating the samples. For the determination of any given amino acid in urine, the urine was diluted as necessary and brought to a pH of 7.0. Urine from mice fed 10 per cent of casein was diluted approximately 10-fold, while urine from mice fed 50 per cent of casein was diluted 50-fold. Rat urine, which usually contains one-half or less of any given amino acid than urine from mice on a comparable diet, was diluted about one-half as much as the corresponding mouse urine. In any determination, amounts of the diluted urine, ranging from 0.1 to 1.0 ml., were added to 1.0 ml. of the medium and the volume of the mixture was brought to 2.0 ml. with distilled water. Accompanying tubes containing graded supplements of the amino acid being determined yielded the standard curve on which calculations were based. The standard usually consisted of synthetic *dl* acids, but calculations were based on the *l* isomer only (1, 14, 16); for arginine, cystine, glutamic acid, lysine, and tyrosine, the *l* forms were used directly.

In general, the dilutions chosen were such that all of the aliquots used in the determinations (from 0.1 to 1.0 ml. of diluted urine) yielded values corresponding to the usable part of the standard curve. With *Leuconostoc mesenteroides*, only amounts corresponding to the lower portion of the curve were used for the calculations. The organism, medium, and effective range of concentration used for each amino acid are listed in Table II. The

standard curve for every acid was completely regular and approximately linear for the lower two-thirds of the concentration ranges listed.

TABLE II

Organisms and Media Used in Determination of Specific Amino Acids

Amino acid	Organism used	Standard curve, range per tube	Medium
		γ	
Arginine	*Streptococcus faecalis*	0–20	I, synthetic
Aspartic acid	*Leuconostoc mesenteroides*	0–40	III, synthetic, phosphate increased 4 times
Cystine	" "	0–10	V
Glutamic acid	*S. faecalis*	0–60	II,* synthetic
	Lactobacillus arabinosus	0–60	I,* "
Histidine	*S. faecalis*	0–20	" "
Isoleucine	*Lactobacillus arabinosus*	0–25	I, "
Leucine	" "	0–25	" "
Lysine	*Leuconostoc mesenteroides*	0–30	III, "
	S. faecalis	0–30	II, "
Methionine	*Lactobacillus arabinosus*	0– 5	V
	S. faecalis	0–10	" citrate buffer
Phenylalanine	*Lactobacillus arabinosus*	0–10	I, synthetic
Proline	*Leuconostoc mesenteroides*	0–20	III, "
Serine	" "	0–20	" "
Threonine	*S. faecalis*	0–20	II, "
Tryptophane	*Lactobacillus arabinosus*	0– 2	IV
	S. faecalis	0– 2	" citrate buffer
Tyrosine	*Leuconostoc mesenteroides*	0–20	III, synthetic
	Lactobacillus arabinosus	0–20	I, "
Valine	" "	0–25	" "

* 10 γ of glutamine and 6 mg. of NH_4Cl added per tube.

Results

All of the amino acids studied were found in rat urine, although the amounts were often small, ranging from 0.17 mg. of microbiologically available tryptophane to 3.8 mg. of glutamic acid excreted daily by rats on 10 per cent of casein. The differences between acids appeared to be primarily a reflection of the composition of the protein ingested, casein, in which glutamic acid happens to be particularly high. The percentages of ingested amino acid excreted in the urine ranged from 0.3 per cent for lysine to 1.6 per cent for proline. The apparent percentage for the excretion of cystine was still higher, but most of this amino acid was fed in the free form rather than combined as protein, and hence the data on cystine may not be directly comparable with the other values.

Rats fed 50 per cent of casein excreted larger amounts of each amino acid than rats on 10 per cent of casein. For example, 0.37 mg. of histidine was excreted daily on 10 per cent of casein, as compared to 1.16 mg. daily on 50 per cent of casein (Table III), although the urinary excretion of aspartic acid was not very different on the two levels of casein, 0.26 and 0.32 mg. per day respectively. The percentages of the ingested amino acids excreted by the rat were invariably lower on the high protein diet than on the low

TABLE III

*Microbiologically Available Amino Acids in Urine of Rats Fed 10 or 50 Per Cent of Casein**

Amino acid	Amino acid content of casein	10 per cent casein diet			50 per cent casein diet		
		Amino acids ingested daily	Amino acids excreted daily	Ingested amino acid excreted	Amino acids ingested daily	Amino acids excreted daily	Ingested amino acid excreted
	per cent	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Arginine.............	3.7	46.4	0.42	0.91	229	1.16	0.50
Aspartic acid........	6.3	79.0	0.26	0.32	392	0.33	0.08
Cystine*............	0.36	(17.1)	(0.42)	(2.46)	(35)	(0.69)	(1.98)
Glutamic acid	21.5	269.6	3.80	1.40	1334	11.59	0.87
Histidine...........	2.7	33.9	0.37	1.09	169	1.16	0.69
Isoleucine	6.5	81.5	0.68	0.83	403	1.44	0.36
Leucine	9.9	124.2	1.46	1.17	594	4.33	0.73
Lysine..............	7.6	95.5	0.30	0.31	472	1.04	0.22
Methionine..........	3.0	37.6	0.26	0.69	186	0.50	0.30
Phenylalanine	5.2	65.2	0.52	0.79	322	1.69	0.52
Proline.............	8.2	102.8	1.64	1.61	509	4.55	0.89
Serine	6.4	80.2	0.94	1.17	397	0.73	0.18
Threonine...........	3.7	46.5	0.74	1.58	230	2.28	1.01
Tryptophane	1.2	15.1	0.17	1.15	75	0.59	0.78
Tyrosine............	5.6	70.2	0.67	0.97	435	2.44	0.56
Valine..............	6.2	84.0	0.51	0.60	415	0.87	0.21

* The diet contained 0.1 per cent $l(-)$-cystine, in addition to that contained in the casein. Since some may have spilled, values for cystine are in parentheses.

protein diet. Thus, 1.4 per cent of the glutamic acid ingested in the low casein diet appeared in the urine in contrast to 0.87 per cent of that ingested on the high casein diet. For a typical essential amino acid, histidine, the percentages excreted were 1.1 and 0.7 per cent of the amounts ingested on the low and high protein diets, respectively. The percentages of ingested aspartic acid and serine excreted by rats on the high protein diet were particularly low, 0.08 and 0.18 per cent, respectively. A few observations on urine from rats fed 20 per cent of casein indicated that the percentages of amino acids excreted lay between those obtained on the diet containing 10

and 50 per cent of casein respectively. Taken collectively, neither the amounts nor the percentages of the non-essential amino acids excreted were significantly different from those for the essential amino acids (Table III).

In line with our previous observations on tryptophane (3), a much larger percentage of the other amino acids ingested appeared in the free or microbiologically available form in mouse urine than in rat urine. On the 10 per

TABLE IV

Microbiologically Available Amino Acids in Urine from Mice Fed 10 or 50 Per Cent of Casein*

Amino acid	10 per cent casein diet			50 per cent casein diet		
	Amino acid ingested† daily	Amino acid excreted daily	Ingested amino acid excreted	Amino acid ingested† daily	Amino acid excreted daily	Ingested amino acid excreted
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Arginine..............	12.03	0.66	5.50	60.1	1.97	3.28
Aspartic acid..........	20.48	0.20	1.01	102.8	1.21	1.18
Cystine*..............	(4.42)	(0.34)	(7.80)	(9.1)	(1.02)	(11.25)
Glutamic acid.........	69.88	2.52	3.61	349.4	11.25	3.22
Histidine..............	8.80	0.27	3.15	44.9	1.21	2.69
Isoleucine.............	21.13	0.46	2.20	105.6	2.71	2.57
Leucine...............	32.18	0.96	3.00	160.9	4.85	3.02
Lysine................	24.70	0.53	2.14	123.2	2.52	2.04
Methionine............	11.38	0.25	2.22	56.8	1.26	2.20
Phenylalanine.........	16.90	0.46	2.74	84.50	1.79	2.12
Proline...............	26.65	1.03	3.88	133.2	5.25	3.94
Serine................	20.80	0.84	4.06	104.0	3.30	3.17
Threonine.............	12.03	0.51	4.25	60.0	2.38	3.96
Tryptophane..........	3.90	0.09	2.39	19.5	0.44	2.28
Tyrosine..............	18.20	0.35	1.98	91.0	1.79	1.98
Valine................	21.78	0.54	2.48	108.8	2.68	2.47

* The diet contained 0.1 per cent $l(-)$-cystine, in addition to that contained in the casein. Since some may have spilled, values for cystine are in parentheses.

† Calculated from the percentages of amino acids in casein listed in Table III.

cent casein diet, the percentages appearing in mouse urine ranged from 1.0 per cent for aspartic acid to 5.5 per cent for arginine (Table IV). On the 50 per cent casein diet, the percentages of excretion ranged from 1.2 per cent for aspartic acid to 3.9 per cent for threonine. All other amino acids yielded intermediate values, except cystine, which was present in the diet in the free form.

The similarity in the percentage of excretion of amino acids by mice on the two levels of protein (Table IV) may be related to the observation that adult mice apparently thrive on both diets (4). It is of interest that mice

not only excrete a higher percentage of ingested protein as free amino acids than do rats, but that the amounts of microbiologically available amino acids excreted daily on a given level of protein were frequently greater for a 25 gm. mouse than for a rat weighing nearly 10 times as much. Thus on the high protein diet, mice excreted 2.0 mg. of arginine daily as compared to 1.16 mg. for rats. For aspartic acid, the figures were 1.2 mg. for mice and only 0.33 mg. for rats. For all other amino acids, the amounts of free acid excreted by the mice were at least as great or greater than those excreted by the rat (Tables III and IV). This difference between species has already been the subject for speculation in the case of tryptophane (3). The higher percentages of amino acids excreted by the mouse might imply a lower efficiency of utilization, possibly associated with the fact that the mouse ingests much larger amounts of food in terms of its own body weight than does the rat. The difference may also be related to the higher met-

TABLE V

α-Amino Nitrogen Excreted Daily by Rats and Mice, As Determined by Various Methods

	Calculated from microbiological determination	Copper method	Van Slyke HNO₂	Formol
	$mg.$	$mg.$	$mg.$	$mg.$
Rats on 10% casein............	1.6	2.6	2.95	11.0
" " 50% "	4.1	7.5	29.1	53.3
Mice " 10% "	1.2	2.6	4.43	4.3
" " 50% "	5.4	6.1	21.3	26.2

abolic rate of the mouse. The major difference, however, is that rat urine contains a higher proportion of peptides (see below).

α-Amino Nitrogen—α-NH₂ nitrogen in the pooled samples of urine was determined by formol titration (17), by the HNO₂ method of Van Slyke (18), and by the copper method of Pope and Stevens (19, 20), and the results were compared with those calculated from the total α-amino N in the amino acids determined microbiologically. For purposes of calculation the percentage excretion of ingested glycine and alanine was assumed to be the same as the average for the other amino acids in any given specimen of urine.

Each of the chemical determinations yielded the same qualitative results as those obtained microbiologically (Table V); namely, that the amount of α-amino N excreted in the urine was roughly proportional to the protein content of the diet, and further that the mouse excreted a higher proportion of ingested protein as the free amino acids than the rat.

However, wide discrepancies were observed between the amounts of α-amino nitrogen as determined on any sample of urine by the various methods (Table V). The values obtained by the copper method were roughly double those calculated from microbiological results; the other methods yielded figures for α-amino nitrogen that were 2- to 12-fold those calculated from microbiological results. The formol titration yielded the highest values, the nitrous acid method was next, while the copper method was the lowest. This is in line with the observations of Van Slyke, MacFadyen, and Hamilton (21) and of others (19) that the former two methods respond to substances other than free amino acids, including the terminal groups in peptides.

The presence of peptides in rat and mouse urine was demonstrated by hydrolyzing the urine with 10 per cent HCl for 5 hours at 15 pounds pres-

TABLE VI

Effect of Hydrolysis upon α-Amino Nitrogen and Certain Amino Acids in Urine
The values are measured in mg. per day.

	α-Amino N*		Histidine		Threonine	
	Before	After	Before	After	Before	After
Rats on 10% casein............	2.6	9.9	0.31	0.87	1.91	5.52
" " 50% "	7.5	34.3	1.08	2.98	5.4	13.6
Mice " 10% "	2.6	5.0	0.49	0.76	2.25	3.32
" " 50% "	6.1	12.8	0.69	0.91	4.23	8.05

* Copper method used.

sure in the autoclave (14). Following hydrolysis the α-amino nitrogen content of mouse urine was doubled (Cu method); from 2.6 to 5.0 mg. daily per mouse on 10 per cent of casein, and from 6.1 to 12.8 mg. per day per mouse on 50 per cent of casein (Table VI). The effect of hydrolysis was still more pronounced on rat urine. Rats on 10 per cent of casein excreted 2.6 mg. of "free" α-amino nitrogen daily; after hydrolysis the same specimen contained 9.9 mg. In urine from rats on 50 per cent of casein a daily specimen contained 7.5 mg. before hydrolysis and 34.3 mg. thereafter. In other words, the α-amino nitrogen of rat urine was increased 4-fold by hydrolysis.

Preliminary determinations of individual amino acids revealed that the hydrolysis had increased the amounts that are microbiologically available. For histidine the increases averaged 40 per cent for mouse urine, and 180 per cent for rat urine (Table VI); for threonine the increases averaged 68 and 170 per cent respectively for urine from the two species.

DISCUSSION

That individual amino acids may appear in normal urine has been suggested in several previous studies in which either chemical (22, 23) or microbiological (2, 3) methods of determination were employed. The new results indicate the presence in urine of all of the common amino acids for which suitable analytical methods are available. Presumably glycine, alanine, and hydroxyproline, the acids not determined, are also present.

While some differences between amino acids appear to exist as far as the percentage excretion of ingested acid is concerned, it is also evident from the present results that at least three general conclusions can be drawn: (a) that the amount of microbiologically available amino acids appearing in the urine depends upon the amount ingested as dietary protein; (b) that the mouse excretes more free amino acid than the rat; and (c) that many amino acids appear in urine in a bound form, presumably in peptide linkages.

Chemical methods for α-amino nitrogen in urine yield higher values than those calculated from microbiological determinations of individual amino acids. One possible explanation could be the presence of d-amino acids in urine. Such acids would not be detected by the three strains of organisms used in the present assays (14), although they would of course be recorded in the chemical tests. Certain amino acids of the d series are said to be excreted almost quantitatively when the corresponding dl acid is fed (24, 25). Apparently the mechanism for excreting d acids is efficient, but whether any d acid is formed at all when a natural protein such as casein is metabolized remains to be demonstrated.

Another possibility is that urine contains inhibitors, e.g., urea, that restrict the growth of the assay organism. In the present study, however, the dilution of the urines analyzed was so great that the inhibiting effects were no longer evident. Recoveries of added phenylalanine, for example, ranged from 93 to 107 per cent, and essentially the same values were obtained when the determinations were made over a 10-fold range of dilution, equivalent to 0.01 to 0.1 cc. of rat urine (10 per cent casein) per 2 ml. of assay medium.

The most important cause of the discrepancy between chemical and microbiological results is the presence of peptides in urine, as demonstrated by the increase in α-amino nitrogen and in individual amino acids after hydrolysis. The terminal acids in the peptides probably respond to most of the chemical procedures employed. They might also respond in certain microbiological determinations, although peptides such as glutamylglutamic acid and glutamyltyrosine, which do not replace glutamic acid in the growth of *Lactobacillus arabinosus* (26), are probably more typical of amino

acids in general. It is doubtful whether the method for a single amino acid has been studied adequately for the influence of compounds other than the one being determined, and it might be questioned whether absolute value should be ascribed to the results of microbiological assays on so complex a mixture as urine. On the other hand, the parallelism observed in the present study between the amounts of protein ingested and the amounts of amino acids excreted in microbiologically available form suggests that the assays yield results that are at least roughly quantitative.

SUMMARY

1. Rats and mice were fed semisynthetic diets containing 10 or 50 per cent of casein and the amounts of each of sixteen amino acids in the urine were determined microbiologically. The amounts excreted in microbiologically available form depended upon the amounts ingested as protein. Rats excreted an average of 0.1 to 1.6 per cent of the amounts ingested; mice excreted 1.0 to 5.5 per cent.

2. No significant differences were observed between the amounts and percentages of the non-essential amino acids excreted and those of the essential amino acids.

3. Chemical determinations of α-amino nitrogen yielded significantly higher values than those obtained microbiologically. The presence of peptides in urine was demonstrated by large increases both in α-amino nitrogen and in microbiologically available amino acids after hydrolysis.

BIBLIOGRAPHY

1. Snell, E. E., in Anson, M. L., and Edsall, J. E., Advances in protein chemistry, 85 (1945).
2. Schweigert, B. S., Sauberlich, H. E., and Elvehjem, C. A., *Science*, **102**, 275 (1945).
3. Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A., and Baumann, C. A., *J. Biol. Chem.*, **164**, 213 (1946).
4. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, **157**, 551 (1945).
5. Wesson, L. G., *Science*, **75**, 334 (1932).
6. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, **155**, 183 (1944).
7. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 69 (1946).
8. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
9. Schweigert, B. S., Sauberlich, H. E., Baumann, C. A., and Elvehjem, C. A., *Arch. Biochem.*, **10**, 1 (1946).
10. Toennies, G., *J. Biol. Chem.*, **145**, 667 (1942).
11. Bennett, M. A., and Toennies, G., *J. Biol. Chem.*, **145**, 671 (1942).
12. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **10**, 55 (1946).
13. Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, **163**, 577 (1946).

14. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
15. Riesen, W. H., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 347 (1946).
16. Hegsted, D. M., *J. Biol. Chem.*, **157**, 741 (1945).
17. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, 11th edition, Philadelphia, 717 (1944).
18. Peters, J. B., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 927 (1932).
19. Albanese, A. A., and Irby, V., *J. Biol. Chem.*, **153**, 583 (1944).
20. Pope, C. G., and Stevens, M. F., *Biochem. J.*, **33**, 1070 (1939).
21. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).
22. Albanese, A. A., Holt, L. E., Jr., Frankston, J. E., and Irby V., *Federation Proc.*, **4**, 80 (1945).
23. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **157**, 59 (1945).
24. Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, **160**, 441 (1945).
25. Baur, H., *Helv. physiol. et pharmacol. acta*, **3**, C31-2 (1945); *Chem. Abstr.*, **40**, 1595 (1946).
26. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).

THE PARTIAL REPLACEMENT OF DIETARY PHENYL-
ALANINE BY TYROSINE FOR PURPOSES
OF GROWTH*

By MADELYN WOMACK AND WILLIAM C. ROSE

(*From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana*)

In the preceding paper of this series, Rose and Womack (1) have shown that, under certain specified conditions, the minimum level of phenylalanine which is capable of supporting maximum growth in weanling rats on a tyrosine-free diet is approximately 0.9 per cent. This proved to be true of both $l(-)$- and dl-phenylalanine, and thereby demonstrated that the optical isomers of this amino acid possess, within the limits of accuracy of the method, essentially equal nutritive value for the species in question.

The present paper is concerned with the possibility of replacing part of the dietary phenylalanine by tyrosine. Elsewhere, we have shown that the replacement of practically *all* of the phenylalanine of the food by tyrosine occasions a rapid *loss* in body weight (Womack and Rose (2)). This was interpreted as indicating that the hydroxyl group of tyrosine does not readily undergo reduction *in vivo*. On the other hand, evidence exists (Moss and Schoenheimer (3)) for the reverse change, the oxidation of phenylalanine to tyrosine.[1] In view of this fact, one would expect that the inclusion of tyrosine in the food would lower the phenylalanine requirement by sparing the conversion of the latter into the former. The experiments outlined below confirm this hypothesis, and indicate that approximately half of the phenylalanine may be replaced by its *p*-hydroxy derivative.

EXPERIMENTAL

Young rats served as the experimental subjects, and were housed in individual cages. Food and water were kept in the receptacles at all times. Thus, the animals were able to eat and drink *ad libitum*. The amino acids which furnished the nitrogen of the diets were purified invariably until they yielded correct analytical values.

The amino acid mixture (Mixture XX-b) was identical in composition

---

[1] Additional evidence for the synthesis of tyrosine *in vivo* will be presented in a later paper.

with that employed in the preceding paper (Rose and Womack (1)). It was devoid of both phenylalanine and tyrosine but contained seventeen amino acids. The basal diet was Diet 2 of Rose and Womack (1). It carried 16.7 per cent of Mixture XX-b, equivalent to 10.5 per cent of physiologically active amino acids. When phenylalanine and tyrosine were incorporated in the food, they replaced an equal weight of dextrin. The slight variations in dietary nitrogen thereby occasioned were without significance in the final results.

When the investigation herein described was in progress, attention was also being directed toward an improvement in the make-up of the pills used as sources of the water-soluble vitamins. Before crystalline vitamins became available in abundance, *adequate* supplementation of amino acid diets, without the introduction of too much nitrogen of an unknown nature, presented an almost insuperable difficulty. In the current study, three formulas were used in preparing the vitamin supplements. For convenience in reference each type is numbered, and its composition is given below.

Daily Vitamin Intakes

Vitamin Supplement 1—150 mg. of milk concentrate and 100 mg. of tikitiki extract.

Vitamin Supplement 2—25 mg. of ryzamin-B, 22 mg. of wheat germ oil, and 20 γ each of thiamine hydrochloride, riboflavin, and nicotinic acid.

Vitamin Supplement 3—20 γ each of thiamine hydrochloride, nicotinic acid, and pyridoxine hydrochloride, 40 γ of riboflavin, 100 γ of calcium *d*-pantothenate, 10 mg. of choline chloride, 3 mg. of *p*-aminobenzoic acid, 10 mg. of inositol, 50 mg. of wheat germ oil, and 25 mg. of Wilson's liver powder 1:20.

The figures represent *daily* intakes. In each type, the ingredients specified were mixed with a little dextrin, moistened with water, and rolled into pills. Two pills, each containing half of the daily allotment, were administered to each animal daily. No detectable differences were observed in the growth effects of Supplements 1 and 2 under the conditions in which they were employed. Supplement 3 is superior to the others, and consequently permits more rapid gains in weight. However, all comparisons between different levels of phenylalanine or tyrosine were made with a single vitamin supplement, as indicated in Tables I to III.

The first series of tests had to do with the influence of different levels of phenylalanine upon growth when the diet contained a liberal amount (0.8 per cent) of tyrosine. Three litters of animals were employed for this purpose, and the results are summarized in Table I. For the convenience of the reader, the average gain on each level of phenylalanine is

shown, although we recognize fully that small differences in such averages are not significant since the number of animals in each group is necessarily small.

TABLE I

Effect of Phenylalanine on Growth When Diet Contains 0.8 Per Cent of l(−)-Tyrosine
Each experiment covered 28 days.

Litter No.	Rat No. and sex	Total gain in weight	Average	Total food intake	Amino acid supplement, *dl*-phenylalanine
		gm.		*gm.*	*per cent*
1*	3583 ♂	−4		51	0.1
	3584 ♀	−3		61	0.1
	3585 ♀	−4	−3.7	53	0.1
	3586 ♂	24		87	0.3
	3587 ♀	23		84	0.3
	3588 ♀	33	26.7	114	0.3
	3589 ♂	53		136	0.5
	3590 ♀	46		151†	0.5
	3591 ♀	54	51.0	172†	0.5
2*	3652 ♀	51		144	0.4
	3653 ♂	44		135	0.4
	3654 ♂	45	46.7	148	0.4
	3655 ♀	44		133	0.5
	3656 ♂	45		138	0.5
	3657 ♂	42	43.7	148†	0.5
	3658 ♀	36		133	0.7
	3659 ♂	55		153	0.7
	3660 ♂	41	44.0	128	0.7
3*	5112 ♀	47		115	0.4
	5113 ♀	48		134†	0.4
	5114 ♂	48		123	0.4
	5115 ♂	51	48.5	130	0.4
	5116 ♀	51		137	0.5
	5117 ♀	46		131	0.5
	5118 ♀	48		146	0.5
	5119 ♂	53	49.5	159†	0.5
	5120 ♀	51		148	0.7
	5121 ♀	50		146	0.7
	5122 ♂	52	51.0	147	0.7

* Vitamin Supplement 1.
† The animal scattered food; the recorded intake is probably too high.

An examination of the data in Table I reveals several interesting facts. In Litter 1, 0.1 per cent of phenylalanine was insufficient to prevent *losses* in weight despite the presence in the diet of 0.8 per cent of tyrosine. This confirms the earlier report of Womack and Rose (2) that tyrosine is in-

capable of replacing phenylalanine in the organism of the rat. With higher levels of dietary phenylalanine growth occurred, and the magnitude of the gain was distinctly greater with 0.5 than with 0.3 per cent. In Litters 2 and 3, the gains upon diets containing 0.5 and 0.7 per cent of

TABLE II

Growth on Different Proportions of Phenylalanine and Tyrosine

Each experiment covered 28 days.

Litter No.	Rat No. and sex	Total gain in weight	Total food intake	Amino acid supplements
		gm.	*gm.*	
4*	3825 ♀	40	132	1.0% *dl*-phenylalanine
	3826 ♂	34	111	1.0% "
	3827 ♂	40	121	1.0% "
	3828 ♂	54	140	1.0% "
Average......		42.0		
	3829 ♀	45	126	1.0% *dl*-phenylalanine + 0.6% *l*(−)-tyrosine
	3830 ♂	48	138	1.0% " + 0.6% "
	3831 ♂	33	108	1.0% " + 0.6% "
	3832 ♂	41	124	1.0% " + 0.6% "
Average......		41.8		
5†	5762 ♀	14	89	0.5% *dl*-phenylalanine
	5763 ♀	11	84	0.5% "
	5764 ♂	14	94	0.5% "
	5765 ♂	20	107	0.5% "
Average......		14.8		
	5766 ♀	55	153	0.5% *dl*-phenylalanine + 0.8% *l*(−)-tyrosine
	5767 ♀	55	147	0.5% " + 0.8% "
	5768 ♀	66	166	0.5% " + 0.8% "
	5769 ♂	79	200	0.5% " + 0.8% "
Average......		63.8		

* Vitamin Supplement 2.
† Vitamin Supplement 3.

phenylalanine were not significantly superior to the gains upon 0.4 per cent. Thus, *when sufficient tyrosine is included in the food the phenylalanine requirement for maximum increases in weight is reduced about half* (from 0.9 per cent to approximately 0.4 per cent).

Further evidence as to the rôle of tyrosine in growth is shown by the

data in Table II. When the diet contained an adequate amount of phenylalanine (1.0 per cent), as was the case with Litter 4, the addition of tyrosine failed to accelerate the rate of gain. Thus, *tyrosine stimulates growth only when phenylalanine is furnished in suboptimal amounts.* On the other hand, a diet carrying slightly more than half the phenylalanine required in the absence of tyrosine (Litter 5, Table II) permitted very poor growth as compared to the gains when tyrosine also was included.

<div align="center">TABLE III</div>

Effect of Tyrosine on Growth When Diet Contains 0.4 Per Cent of dl-Phenylalanine
Each experiment covered 28 days.

Litter No.	Rat No. and sex	Total gain in weight	Average	Total food intake	Amino acid supplement, $l(-)$-tyrosine
		gm.		*gm.*	
6*	3841 ♀	20		121	0.1%
	3842 ♂	24		111	0.1%
	3843 ♂	21	21.7	105	0.1%
	3844 ♀	35		140†	0.3%
	3845 ♂	39		134	0.3%
	3846 ♂	37	37.0	134†	0.3%
	3847 ♀	45		128	0.5%
	3848 ♂	44		174†	0.5%
	3849 ♂	44	44.3	130†	0.5%
7*	3897 ♀	37		130†	0.3%
	3898 ♂	42		148	0.3%
	3899 ♂	46	41.7	161	0.3%
	3900 ♀	41		152†	0.4%
	3901 ♂	39		114	0.4%
	3902 ♂	42	40.7	129	0.4%
	3903 ♀	45		169†	0.5%
	3904 ♂	45		145†	0.5%
	3905 ♂	46	45.3	154†	0.5%

* Vitamin Supplement 2.
† The animal scattered food; the recorded intake is probably too high.

· In the final series of tests the procedure was the reverse of that employed in the experiments recorded in Table I. Instead of furnishing a constant excess of tyrosine with varying levels of phenylalanine, the diet now contained 0.4 per cent of phenylalanine with differing percentages of tyrosine. The results upon two litters are presented in Table III. In Litter 6, each increment of 0.2 per cent in the tyrosine content of the food induced an increase in growth. In Litter 7, the tyrosine increments were 0.1 per cent, and the differences in total gains were not so pronounced. But even here, 0.5 per cent appears to be slightly superior to either of the

lower levels. Thus, the supplementing action of tyrosine in the presence of a partial deficiency of phenylalanine is again manifested. Evidently, the relationship of these amino acids to each other, in so far as growth is concerned, is precisely the same as that which exists between methionine and cystine, except that in the latter case only about one-sixth of the minimum methionine requirement can be served by cystine (cf. Womack and Rose (4)).

SUMMARY

Experiments involving the use of otherwise adequate diets containing varying proportions of phenylalanine and tyrosine have demonstrated that in the rat tyrosine is capable of stimulating growth only when phenylalanine is furnished in suboptimal amounts.

In this species, the inclusion of appropriate quantities of tyrosine in the food reduces the minimum phenylalanine requirement for maximum gains, as determined in the absence of tyrosine, from about 0.9 per cent of the diet to approximately half this figure.

BIBLIOGRAPHY

1. Rose, W. C., and Womack, M., J. Biol. Chem., **166,** 103 (1946).
2. Womack, M., and Rose, W. C., J. Biol. Chem., **107,** 449 (1934).
3. Moss, A. R., and Schoenheimer, R., J. Biol. Chem., **135,** 415 (1940).
4. Womack, M., and Rose, W. C., J. Biol. Chem., **141,** 375 (1941).

THE ANTAGONISM OF SULFONAMIDE INHIBITION OF CERTAIN LACTOBACILLI AND ENTEROCOCCI BY PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

By J. O. LAMPEN and M. J. JONES

(From the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut)

Several lines of evidence have indicated an interrelation between pteroic acid derivatives (*Lactobacillus casei* factor, folic acid, etc.) and sulfonamides in bacterial metabolism. Teply *et al.* (1) demonstrated that the inhibition of *Lactobacillus arabinosus* by sulfapyridine was reversed by a factor in liver extract which resembled folic acid. Also, Miller (2) found the synthesis of folic acid by *Escherichia coli* to be inhibited by sulfanilamide. Finally, Angier *et al.* (3) recently demonstrated that the PABA[1] moiety occurs in the pteroic acid molecule. PABA is known to antagonize sulfonamides competitively (4).

We have studied the effect of sulfonamides on the metabolism of pteroic acid derivatives both in organisms requiring the preformed molecules for growth and in those able to synthesize their own requirements. We have also investigated the possible antagonism of sulfonamides by these compounds and the mechanism of this antagonism where observed.

In this paper, the results obtained with certain lactobacilli and enterococci are presented. A preliminary report of a portion of this work has previously been published (5).

EXPERIMENTAL

Stock Cultures and Inocula—The various lactobacilli and enterococci[2] were maintained as stabs in "A. C. broth experimental" (Difco) with 2 per cent agar and added liver extract (6), or in a meat infusion medium containing (per 100 ml.) agar 1.5 gm., tryptone (Difco) 1.0 gm., glucose 0.1

---

[1] The following abbreviations are used throughout for the sake of brevity: PABA, *p*-aminobenzoic acid; PABG, *p*-aminobenzoyl-*l*(+)-glutamic acid; SA, sulfanilamide; SP, sulfapyridine; ST, sulfathiazole; SD, sulfadiazine.

[2] *Streptococcus faecalis* R is No. 8043, *Lactobacillus casei* is No. 7469, and *Lactobacillus plantarum* is No. 4943 of the American Type Culture Collection. *Streptococcus faecalis* (Ralston) was obtained from Dr. E. H. Spaulding of Temple University, Philadelphia, Pennsylvania; the other enterococci from Dr. C. F. Niven, Jr., of Cornell University, Ithaca, New York; and *Lactobacillus delbrückii* LD5 and *Lactobacillus bulgaricus* 05 from Dr. J. L. Stokes of Merck and Company, Inc., Rahway, New Jersey.

gm., K_2HPO_4 0.2 gm., $CaCO_3$ 0.3 gm., and Bacto beef extract, 0.1 gm. All of the organisms grew well on both media. The meat infusion agar appeared slightly superior for the enterococci, however, and it was used for all cultures in the later stages of the work.

The organisms to be used as inocula were generally grown overnight on the A. C. broth-liver extract medium, centrifuged, washed with saline-phosphate buffer (6), and resuspended in the original volume of buffer. This suspension was diluted 100-fold and 1 drop added per 10 ml. of medium. A detectable carryover of pteroic acid derivatives by this pro-

TABLE I

Constituents of Basal Medium I

	Per liter		Per liter
	gm.		*mg.*
Glucose	10	Guanine hydrochloride	5
Sodium acetate	6	Uracil	5
Casein hydrolysate*	5	Thiamine hydrochloride	1
l-Tryptophane	0.1	Riboflavin	1
l-Cystine	0.1	Calcium pantothenate	1
Asparagine	0.1	Nicotinic acid	1
	ml.	Pyridoxine	2
Salts A†	5		*γ*
" B†	5	Biotin	5
	mg.	pH to 6.6–6.8	
Xanthine	5		
Adenine sulfate	5		

* "Vitamin-free" casein hydrolysate (General Biochemicals, Inc.). When necessary, this material was freed of *p*-aminobenzoic acid by stirring for 30 minutes at pH 3.5 with 1 per cent Darco G-60.

† Salts A contains 25 gm. of K_2HPO_4 and 25 gm. of KH_2PO_4 dissolved in 250 ml. of water. Salts B contains 10 gm. of $MgSO_4 \cdot 7H_2O$, 0.5 gm. of NaCl, 0.5 gm. of $FeSO_4 \cdot 7H_2O$, and 0.5 gm. of $MnSO_4 \cdot 4H_2O$ dissolved in 250 ml. of water.

cedure occurred only with *Streptococcus liquefaciens* 815 and *Streptococcus zymogenes* 26C1, both of which are able to grow without preformed pteroyl-glutamic acid (see below). Satisfactory organisms for inocula of these strains were grown on Basal Medium I (Table I).

Media and Techniques—The constituents of Basal Medium I are listed in Table I. This medium was employed in all experiments unless otherwise specified. The purines and uracil were omitted from this to give Basal Medium II. Unless otherwise indicated, all supplements were sterilized by autoclaving them after solution in the medium. All cultures were incubated at 37°. When turbidity was used as a growth criterion, this was

measured with a photronreflectometer (7). With our instrument, a reading of 40 is equivalent to an optical density of 0.056 and the relation between galvanometer deflection and cell concentration is linear up to a reading of about 80.

All values for the minimal effective concentrations are the concentrations required for half maximal inhibition. Half maximal inhibition indicates the reduction of the turbidity or acid production at the given time to one-half of that obtained in control tubes. Half maximal antagonism indicates the production of one-half of the control turbidity or acid production in the presence of amounts of inhibitor which would otherwise prevent visible growth.

Materials—Synthetic pteroylglutamic acid[3] was used in all experiments reported here. The preparations of pteroyltriglutamic acid were isolated from a fermentation liquor. They were crystalline and about 80 per cent pure, with the impurities largely ash. Both materials contained 0.5 to 1.0 per cent of free arylamine (as PABA[4]). The quantities of the triglutamic acid used are given in terms of the pure compound. Only one sample of pteroic acid was available. This contained 6 per cent free arylamine (as PABA[5]). Our sample of the glutamic acid polypeptide of PABA, isolated by Ratner *et al.* (8), contained 7.0 per cent of bound PABA.[6]

Results

Effect of Sulfonamides on Streptococcus faecalis R and Lactobacillus casei—In preliminary experiments it was noted that the growth of *Streptococcus faecalis* R on a basal medium containing 0.003 γ of pteroyltriglutamic acid per ml. was not prevented by 1280 γ of SD per ml., although the growth did appear to be somewhat slower. The growth of *Lactobacillus casei*, *L. bulgaricus* 05,[7] *L. delbrückii* LD5,[7] and *L. plantarum* was unaffected by the same concentration of SD in a medium containing 0.0001 γ of pteroyl-
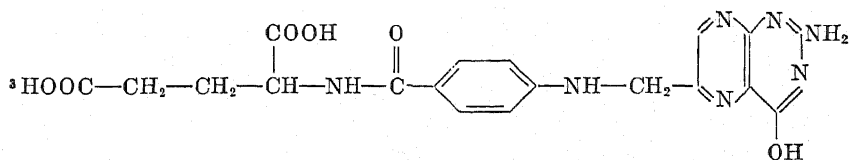
[3] N-[4-{[(2-Amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]glutamic acid (3).

[4] Dr. B. L. Hutchings and Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc., furnished the samples of pteroic acid and its derivatives and information concerning them.

[5] Determined in this Laboratory by Miss Barbara Wadsworth.

[6] Obtained from Dr. D. E. Green of Columbia University, New York.

[7] These organisms have recently been reported identical with *Lactobacillus casei* (9).

triglutamic acid per ml. All of these organisms were similarly resistant to SD in the presence of 1 γ of thymine per ml.

A detailed study was made of the utilization by *Streptococcus faecalis* R of compounds related to pteroic acid and of the effect of sulfonamides on the growth of this organism. The concentrations of the various substances required for half maximal growth at 24 hours are listed in Table VII. This strain showed a variable lag in growth in the presence of suboptimal levels of pteroic acid. For half maximal turbidity at 16 hours 0.0004 to 0.0008 γ per ml. was usually required. When growth was measured after 3 days, however, this compound appeared to be as active as pteroylglutamic acid. This lag was not decreased by the addition of 0.2 mg. of *dl*-alanine and 0 3 mg of norit-treated peptone per ml., as suggested by Teply and

TABLE II

Response of Streptococcus faecalis R to Compounds Related to Pteroic Acid in Presence of Sulfadiazine

Turbidity readings (see the text) after 16 hours incubation.

Compound		Sulfadiazine, γ per ml.			
		0	10	100	1000
	γ per ml.				
Pteroyltriglutamic acid	0.003	14	16	24	6
	0.006	48	44	50	36
Pteroylglutamic acid	0.0001	39	32	33	23
	0.0003	74	76	73	57
Pteroic acid	0.0002	69	60	58	0
	0.0006	99	92	91	75
Thymine	0.5	34	39	43	34
	3.0	44	43	37	45

Elvehjem (10) in their assay procedure. The rate of growth was optimal in the presence of excess pteroic acid (0.01 γ per ml.). The quantities of the other compounds needed for half maximal turbidity or acid production after 64 hours were generally slightly less than at 24 hours. The highest growth level obtained with thymine after 64 hours ranged between the half maximum and maximum in various experiments.

The data of Table II show that the turbidity attained by *Streptococcus faecalis* R on the pteroic acid derivatives after 16 hours was not lowered significantly by the presence of 10 or 100 γ of SD per ml. With all of the compounds except thymine, the turbidities obtained were reduced by 1000 γ of SD per ml. The lag during growth on pteroic acid was slight in this experiment. However, 1000 γ of SD per ml. prevented for 16 hours the appearance of growth in the presence of 0.0002 γ of pteroic acid per ml.

After 64 hours, about half of the control turbidity had been reached.   Slight inhibition by SD was still evident with the other compounds after 64 hours.

For half maximal acid production after 64 hours, *Lactobacillus casei* required the indicated concentration of any one of the following compounds: pteroyltriglutamic acid 0.00016 γ per ml., pteroylglutamic acid 0.00008 γ per ml., pteroic acid 1 γ per ml., thymine 0.15 γ per ml.   The acid production or turbidity in the presence of thymine alone did not exceed half maximum.   The growth of *Lactobacillus casei* in the presence of these

TABLE III

Response of Lactobacillus casei to Compounds Related to Pteroic Acid in Presence of Sulfadiazine

Compound		Basal medium No.	Hrs. incubation	Turbidity readings				
				Sulfadiazine, γ per ml.				
				0	100	300	1000	2000
	γ *per ml.*							
Pteroyltriglutamic acid	0.0002	I	24	18	15		14	
	0.0002	I	40	37	38		32	
Pteroylglutamic acid	0.00005	I	24	11	13		8	
	0.00005	I	40	23	25		20	
Pteroic acid	1.0	I	24	11	12		9	
	1.0	I	40	25	27		22	
Thymine	0.15	I	24	6	6		4	
	0.15	I	40	33	30		28	
Pteroyltriglutamic acid	0.00024	II	42	18		12	9	9
	0.00024	II	89	54		47	37	34
Pteroylglutamic acid	0.0001	II	42	6		11	12	5
	0.0001	II	89	36		40	30	23
Pteroic acid	1.0	II	42	6		9	8	3
	1.0	II	89	19		22	18	13

substances was affected very little by 100 γ per ml. of SD in a medium containing the purines and uracil (Basal Medium I).   Some inhibition occurred at 1000 γ of SD per ml. (Table III).

Snell and Mitchell (11) reported that *Lactobacillus casei* was inhibited for 36 hours on a purine-free medium by 1000 γ of SA per ml.   This inhibition was partially antagonized by PABA or by the purines.   We tested the effect of 300 to 2000 γ of SD per ml. on the response of this organism to the pteroic acid compounds on a similar medium (No. II).   The data of Table III show that growth was reduced, although not completely prevented, by 1000 to 2000 γ of SD per ml.   Occasional stimulation of growth has been observed at subinhibitory sulfonamide levels both with this organism and with *Streptococcus faecalis* R.

The longer growth periods used with Basal Medium II (Table III) were necessitated by the fact that the growth on the pteroic acid derivatives was much slower in the absence of the purines. Approximately the same turbidity values were ultimately reached, however. Table IV presents the data obtained with pteroyltriglutamic acid which are characteristic of the results with all three compounds. As has previously been reported (12, 13), the purines were essential rather than stimulatory for the growth of either organism on thymine, at least over the 72 hour period. Adenine, guanine, xanthine, or hypoxanthine at 10 γ per ml. could replace the com-

TABLE IV

Effect of Purines on Response of Lactobacillus casei to Pteroyltriglutamic Acid and to Thymine

Pteroyltriglu-tamic acid	Thymine	Turbidity readings			
		Basal Medium II		Basal Medium II + purines*	
		24 hrs.	72 hrs.	24 hrs.	72 hrs.
γ per ml.	γ per ml.				
0.00005		0	3	1	2
0.00020		2	17	7	12
0.0010		5	69	21	69
0.0050		3	82	34	86
	0.05			1	7
	0.15			6	33
	0.20	0	1	17	40
	1.0	0	0	15	37
	10.0	0	0		

* 10 γ each of adenine, guanine, and xanthine per ml.

plete purine mixture for either organism, whereas uracil or cytosine gave only a slight variable response.

An apparent antagonism by purines of the effects of 1000 γ of SD per ml. could readily be obtained after 24 to 48 hours (Table III) because of the stimulation of the rate of growth by these substances. When controls containing purines were used for comparison, however, the effect of the SD was seen to be essentially unchanged. PABA (1 γ per ml.) and *dl*-methionine (100 γ per ml.), which did not stimulate growth in the presence or absence of purines, showed no apparent SD antagonism. Our results are not entirely analogous to those of Snell and Mitchell, however, since the growth of their culture at 24 hours was not increased by the addition of purines. Their strain was also more sensitive to the effects of the high sulfonamide concentrations.

From the data with *Streptococcus faecalis* R and *Lactobacillus casei* we

conclude that the growth of these organisms and therefore their utilization of pteroic acid derivatives and thymine is essentially normal in the presence of therapeutic levels of SD. Under our conditions, high concentrations of SD exert some non-specific inhibitory action which, at least with *Lactobacillus casei*, is not antagonized by PABA, methionine, or purines.

Sulfonamide Antagonism with Streptococcus faecalis (Ralston)—The data of Table V show that this organism, in contrast to *Streptococcus faecalis* R, grew slowly on Basal Medium I without added pteroic acid derivatives and that both the rate of growth and final growth attained were increased by the addition of pteroyltriglutamic acid. A number of other related compounds were stimulatory. The concentrations of these producing a 50 per cent increase in turbidity at 24 hours are listed in Table VII. It ap-

TABLE V

Effect of Pteroyltriglutamic Acid on Growth of Streptococcus faecalis (Ralston)

Pteroyltriglutamic acid	Growth response*		
	16 hrs. (T.)	64 hrs. (T.)	64 hrs. (A.)
γ per ml.			
0.0	5	24	1.65
0.0008	6	31	1.65
0.0024	7	26	1.50
0.008	18	43	2.45
0.08	23	55	3.50

* T., turbidity readings; A., ml. of 0.1 N acid per 10 ml.

pears, therefore, that this organism synthesizes its own supplies of PABA and pteroic acid derivatives at a suboptimal rate.

It was found that the organism was extremely sensitive to sulfonamides when growing on Basal Medium I. On the addition of 0.004 γ of pteroyltriglutamic acid or 1 γ of thymine per ml. it became highly resistant to all four sulfonamides tested. This concentration of pteroyltriglutamic acid was later found to be border line and the antagonism was not always complete. The results with these and related compounds are summarized in Table VI. PABA at 0.005 γ per ml. had much less effect that did 0.004 γ of pteroyltriglutamic acid per ml. The result with 500 γ of PABG (14, 15) per ml. was the maximal antagonism obtained with this compound. Xanthopterin was inactive alone and did not increase the antagonism by PABG. A mixture of methionine, serine, glycine, and xanthine (16) was also inactive in this casein hydrolysate medium.

Table VII presents the results of a more detailed study of the antagonism of various levels of SD. Relatively constant ratios of antagonist to SD

were obtained with PABA or PABG over a 1000-fold range of SD concentration. Thus, the antagonism by these substances is competitive. PABG had only about 0.1 per cent of the activity of PABA. The antagonism by pteroyltriglutamic acid, pteroylglutamic acid, and thymine was noncompetitive; *i.e.*, a relatively constant amount was required to antagonize all levels of SD. The pteroic acid sample gave a competitive antagonism.

TABLE VI

Effect of Compounds Related to Pteroic Acid on Sulfonamide Inhibition of Streptococcus faecalis (Ralston)

Sulfonamide	Antagonist		Minimal effective concentration of sulfonamide*	
			16 hrs. (T.)	64 hrs. (T. or A.)
		γ *per ml.*	γ *per ml.*	γ *per ml.*
Sulfadiazine			<0.04	0.08
"	Pteroyltriglutamic acid	0.004	640	>1280
"	" "	0.008	>1280	>1280
"	Thymine	1.0	>1280	>1280
"	p-Aminobenzoic acid	0.005	0.6	5
"	p-Aminobenzoyl-*l* (+) - glutamic acid	0.010	0.04	0.16
"	" "	500	80	320
"	Xanthopterin	500	<0.04	0.08
"	⎧Xanthopterin ⎨+ PABG	500 500	40	320
"	⎧*dl*-Methionine ⎪*dl*-Serine ⎨Glycine ⎩Xanthine	10 5 5 5	0.08	0.16
Sulfanilamide			0.16	1.25
"	Pteroyltriglutamic acid	0.004	320	>1280
Sulfapyridine			0.08	0.3
"	Pteroyltriglutamic acid	0.004	640	1280
Sulfathiazole			0.04	0.04
"	Pteroyltriglutamic acid	0.004	320	640

* T., turbidity as growth criterion; A., acid production as growth criterion.

It can be seen from Table VII that the activity of this sample is approximately that expected from its content of free PABA. No purer samples have been available for a more critical test of its antagonistic action but any inherent activity of the pteroic acid itself must be slight. The amounts of the various compounds required for antagonism after 64 hours on the basis of acid production or turbidity were generally one-third or one-half of those listed in Table VII.

In experiments with SA, a competitive antagonism was also obtained with PABA and PABG. The relative activity of the two compounds was the same as that against SD. This low activity of PABG in both experiments could well be due to contamination with free PABA (15). Pteroyltriglutamic acid at 0.004 to 0.008 γ per ml. gave non-competitive antagonism of 6 to 3000 γ of SA per ml.

The amounts of the various compounds required for growth stimulation with *Streptococcus faecalis* (Ralston) or for growth with *Streptococcus faecalis* R are also listed in Table VII. The amounts of thymine, pteroyl-

TABLE VII

Growth and Antisulfonamide Activity for Streptococcus faecalis Strains

Turbidity at 24 hours was used as the growth criterion. All values are in micrograms per ml.

Compound*	Requirement for half maximal antagonism				Requirement for growth stimulation†	Requirement for half maximal growth
	Sulfadiazine, γ per ml.					
	1	10	100	1000		
p-Aminobenzoic acid........	0.003	0.03	0.3	3	0.001	Inactive
p-Aminobenzoyl-$l(+)$-glutamic acid..............	1	30	300	>300	0.05	"
Pteroyltriglutamic acid	0.004	0.004	0.004	0.008	0.008	0.003
Pteroylglutamic acid.......	0.0003	0.0003		0.0003	0.0001	0.00015
Thymine...................	0.06	0.25	0.25	0.25	0.25	0.3–0.5
Pteroic acid...............	0.03	0.3	10	30	0.001–0.01	0.0003
Free p-aminobenzoic acid in pteroic acid samples...	0.0018	0.018	0.6	1.8		

* The concentration of pteroyltriglutamic acid and thymine was varied in 2-fold steps. The concentrations used with the other compounds were in the ratio of 10:3:1, etc. All antagonists were added as sterile solutions.

† 50 per cent increase in turbidity over that obtained on the basal medium.

glutamic acid, and pteroyltriglutamic acid needed for sulfonamide antagonism approximate those required for growth or for growth stimulation. Thus the utilization of these preformed compounds is apparently unaffected by the presence of the sulfonamide. It is therefore concluded that under these conditions the synthesis of pteroylglutamic acid, etc., from PABA must be the point of sulfonamide inhibition.

Interrelations of Pteroylglutamic Acid and Sulfonamides in Various Enterococci—Pairs of strains of *Streptococcus faecalis*, *S. zymogenes*, and *S. durans* were studied for their sulfonamide sensitivity. One strain of each pair required preformed pteroylglutamic acid, whereas the other could synthesize its own (17). A strain of *S. liquefaciens* able to grow without added

pteroylglutamic acid was also studied. No strains of this organism which require the preformed molecule have been described. The growth requirements and SD sensitivity of these cultures are presented in Table VIII.

None of the cultures requiring the preformed molecule was inhibited by 1280 γ of SD per ml. Strains synthesizing pteroylglutamic acid were inhibited by 0.3 to 0.6 γ of SD per ml., but became insensitive in the presence of the amount of the preformed molecule required for growth by the paired strain. If inocula of *Streptococcus liquefaciens* 815 or *Streptococcus zymogenes* 26C1 grown on A. C. broth were used, the minimal effective concentration of SD in the absence of added pteroylglutamic acid was usually the same as that listed in Table VIII. Light growth occurred, however, even

TABLE VIII

Activity of Sulfadiazine against Enterococcus Strains

All values are expressed in micrograms per ml. Turbidity used as growth criterion.

Culture	Pteroyltriglutamic acid requirement, 64 hrs.		Minimal effective concentration of sulfadiazine, 24 hrs.	
	Half maximal growth	Maximal growth	Basal Medium I	Basal Medium I + 0.008 γ pteroyltriglutamic acid per ml.
Streptococcus faecalis F24.......	0.003	0.008		>1280
" " Gly2......	0	0	0.3	>1280
" *zymogenes* 5C1....	0.003	0.009		>1280
" " 26C1...	0	0	0.6	>1280
" *durans* 24P1...	0.002	0.008		>1280
" " S10	0	0	0.3	>1280
" " 98A....	0.002	0.008		>1280
" *liquefaciens* 815...	0	0	0.6	>1280

with the highest SD concentration. Tests employing inocula grown on Basal Medium I did not show this phenomenon. It seems probable that these strains store sufficient pteroylglutamic acid, etc., during growth on the A. C. broth to support light growth subsequently in the presence of the SD.

It was noted that good growth of the *Streptococcus faecalis* strains F24 and Gly2 required 40 hours at pH 5.5 in contrast to 16 hours at pH 7.2, but that the requirements for pteroyltriglutamic acid were unaltered. The sensitivity to sulfadiazine was also unchanged provided allowance was made for the lag in growth at the lower pH. The apparent sensitivity of *Streptococcus faecalis* to sulfathiazole in urine at pH 5.5 reported by Sung and Helmholz (18) may have been due to the delay in growth occurring at this

pH or to non-specific effects of the high concentrations of sulfathiazole employed. There appears to be no difference in the sulfonamide-pteroyl-glutamic acid interrelations at the lower pH.

Detailed experiments were performed with *Streptococcus zymogenes* 26C1 to check the type of antagonism obtained with the compounds related to pteroic acid (Table IX). The action of PABA and PABG was competitive; PABG was 0.2 to 0.3 per cent as active as PABA. The glutamic acid polypeptide of PABA was almost completely inactive. Ratner *et al.* (8)

TABLE IX

Growth and Antisulfonamide Activity for Streptococcus zymogenes Strains

All values are in micrograms per ml. Turbidity at 24 hours used as growth criterion.

Compound	Strain 26C1				Strain 5C1
	Requirement for half maximal antagonism				Requirement for half maximal growth
	Sulfadiazine, γ per ml.				
	1	10	100	1000	
p-Aminobenzoic acid	0.0005	0.01	0.1	5	Inactive
p-Aminobenzoyl-l(+)- glutamic acid	0.3	3.0	100	>300*	"
Glutamic acid polypeptide of p-aminobenzoic acid	100	>250	>250	>250	"
Pteroyltriglutamic acid	0.002	0.004	0.004	0.008	0.003
Pteroylglutamic acid	0.00005	0.00005	0.0001	0.0001	0.0001
Thymine	0.2	0.2	0.2	0.2	>300†
Pteroic acid	0.03	0.1	1.0	3.0	0.00013
Free p-aminobenzoic acid in pteroic acid sample	0.0018	0.006	0.06	0.18	

* Toxic at concentrations above 300 γ per ml.

† Thymine at 1.5 to 300 γ per ml. supported light growth (approximately 10 per cent of the maximum) which had not increased after 64 hours. The addition of 200 γ of *dl*-alanine and 300 γ of norit-treated peptone per ml. (10) did not improve the response.

have previously reported this substance to have no antisulfonamide activity. Antagonism by pteroylglutamic acid and pteroyltriglutamic acid was non-competitive. The quantities required were similar to those needed by strain 5C1 for half maximal growth. Stokes (19) reported that thymine supported only light growth of strain 5C1. This was confirmed in our tests. The utilization of thymine by strain 26C1 appears normal, however, since a non-competitive antagonism of SD was readily obtained with 0.2 γ of thymine per ml. Pteroic acid showed in general a competitive type of action but its activity against the higher SD levels was definitely greater

than its free PABA content would lead one to expect. This compound may have some inherent antisulfonamide activity with *Streptococcus zymogenes* 26C1. The amounts of the compounds required for antagonism after 64 hours on the basis of acid production or turbidity were again usually one-third or one-half of those listed.

<div align="center">DISCUSSION</div>

The primary action of the sulfonamides under the conditions employed in the present study is directed against the *synthesis* of pteroylglutamic acid via PABA. The effect of the sulfonamide is to produce a pteroylglutamic acid deficiency. The antagonism of this induced deficiency by the addition of the preformed molecule is analogous to the correction of the natural deficiency by the preformed molecule with organisms unable to synthesize pteroylglutamic acid. Several similar non-competitive antagonisms have been demonstrated previously (20–23). It has also been possible to demonstrate non-competitive sulfonamide antagonism by pteroic acid derivatives or thymine with *Lactobacillus arabinosus*.[8]

The relative inactivity of pteroic acid in sulfonamide antagonism cannot be explained at present. With those strains requiring preformed pteroylglutamic acid the conversion of pteroic acid to pteroylglutamic acid which presumably occurs does not appear to be prevented by sulfonamides. This conversion may not occur or may be sulfonamide-sensitive with the strains able to synthesize their own supplies of pteroylglutamic acid.

Pteroylglutamic acid did not antagonize the inhibition by sulfonamide of a series of other organisms, *e.g. Escherichia coli, Staphylococcus aureus, Diplococcus pneumoniae*, etc.[8] None of these organisms required preformed pteroylglutamic acid. This is in accordance with the fact that pteroylglutamic acid is not known to interfere in sulfonamide therapy with most organisms. If the concentration of preformed pteroylglutamic acid in the body fluids is sufficient to support the growth of the enterococci, our data indicate a possible explanation of the clinical insensitivity of these organisms to the sulfonamides (24).

<div align="center">SUMMARY</div>

The growth of *Streptococcus faecalis* R and *Lactobacillus casei* in the presence of pteroic acid derivatives and thymine is relatively insensitive to sulfonamides. At very high sulfonamide concentrations some growth inhibition does occur.

The inhibition by sulfonamides of *Streptococcus faecalis* (Ralston), which apparently synthesizes its own requirements of pteroylglutamic acid at a

---

[8] Lampen, J. O., and Jones, M. J., to be published.

suboptimal rate, is antagonized non-competitively by pteroylglutamic acid, pteroyltriglutamic acid, and thymine. The amounts of these compounds required by the Ralston strain for sulfonamide antagonism or for growth stimulation approximate those required by *Streptococcus faecalis* R for growth.

Enterococcus strains which require preformed pteroylglutamic acid are relatively insensitive to sulfonamides. Those strains able to synthesize this molecule are sensitive when dependent upon this synthesis but are resistant in the presence of the preformed molecule. This antagonism was shown to be non-competitive with *Streptococcus zymogenes* 26C1.

The growth of *Streptococcus faecalis* R or *Lactobacillus casei* in the presence of pteroic acid is resistant to the sulfonamides. This compound possessed relatively slight antisulfonamide activity, however, for *Streptococcus faecalis* (Ralston) or *Streptococcus zymogenes* 26C1.

It is concluded that the primary point of sulfonamide inhibition of the enterococci, under the conditions of this study, is the *synthesis* of pteroylglutamic acid via *p*-aminobenzoic acid.

It is a pleasure to express our thanks to the people who furnished cultures and materials for this investigation.

BIBLIOGRAPHY

1. Teply, L. J., Axelrod, A. E., and Elvehjem, C. A., *J. Pharmacol. and Exp. Therap.*, **77**, 207 (1943).
2. Miller, A. K., *Proc. Soc. Exp. Biol. and Med.*, **57**, 151 (1944).
3. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, **103**, 667 (1946).
4. Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).
5. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **164**, 485 (1946).
6. Roepke, R. R., Libby, R. L., and Small, M. H., *J. Bact.*, **48**, 401 (1944).
7. Libby, R. L., *Science*, **93**, 459 (1941).
8. Ratner, S., Blanchard, M., Coburn, A. F., and Green, D. E., *J. Biol. Chem.*, **155**, 689 (1944).
9. Rogosa, M., *J. Bact.*, **51**, 14 (1946).
10. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157**, 303 (1945).
11. Snell, E. E., and Mitchell, H. K., *Arch. Biochem.*, **1**, 93 (1942).
12. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941).
13. Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **152**, 157 (1944).
14. Auhagen, E., *Z. physiol. Chem.*, **277**, 197 (1943).
15. Williams, R. D., *J. Biol. Chem.*, **156**, 85 (1944).
16. Kohn, H., and Harris, J. S., *J. Bact.*, **44**, 717 (1942).
17. Niven, C. F., Jr., and Sherman, J. M., *J. Bact.*, **47**, 335 (1944).

18. Sung, C., and Helmholz, H. F., *Proc. Staff Meetings Mayo Clin.*, **19,** 577 (1944).
19. Stokes, J. L., *J. Bact.*, **48,** 201 (1944).
20. Fildes, P., *Brit. J. Exp. Path.*, **22,** 293 (1941).
21. Borek, E., Miller, H. K., Sheiness, P., and Waelsch, H., *J. Biol. Chem.*, **163,** 347 (1946).
22. Ivanovics, G., *Z. physiol. Chem.*, **276,** 33 (1942).
23. Nielsen, N., and Johansen, G., *Naturwissenschaften*, **31,** 235 (1943).
24. Spink, W. W., Sulfanilamide and related compounds in general practice, Chicago, 2nd edition, 71 (1942).

PHENYLPYRUVYL DERIVATIVES OF AMINO ACIDS*

By JOSEPH S. FRUTON and MAX BERGMANN†

(From the Department of Physiological Chemistry, Yale University, New Haven, and the Laboratories of The Rockefeller Institute for Medical Research, New York)

Much attention has been given to the biochemical relationships between α-keto acids and α-amino acids, and several enzyme systems, such as the *l*- and *d*-amino acid oxidases and the transaminases, have been shown to catalyze the interconversion of corresponding members of these two groups of substances. Recently, the question has been raised as to whether peptide-like compounds of α-keto acids with amino acids may also undergo enzymatic amination (1–4). Although data on this question are not yet available, the non-enzymatic transfer of an amino group from α-amino-phenylacetic acid to a keto acylamino acid has been demonstrated by Herbst and Shemin (5).

The experimental approach to this problem was initiated in 1930 by the synthesis of pyruvylglycine and pyruvyl-*dl*-phenylalanine (6). These compounds were obtained by the reaction of α,α-diacetaminopropionic acid azlactone with glycine or phenylalanine. Pyruvylglycine and pyruvyl-*dl*-alanine had previously been observed among the products of the hydrolysis of dehydrogenated diketopiperazines (7).

In the present communication there is described an additional method for the synthesis of keto acylamino acids and, in particular, of derivatives of phenylpyruvic acid. This procedure involves treatment of acetyl-dehydrophenylalanylamino acids (8) with dilute hydrochloric acid or a mixture of acetic acid and dilute hydrochloric acid. The reaction may be illustrated by the conversion of acetyldehydrophenylalanyl-*l*-leucine (I) to phenylpyruvyl-*l*-leucine (II).
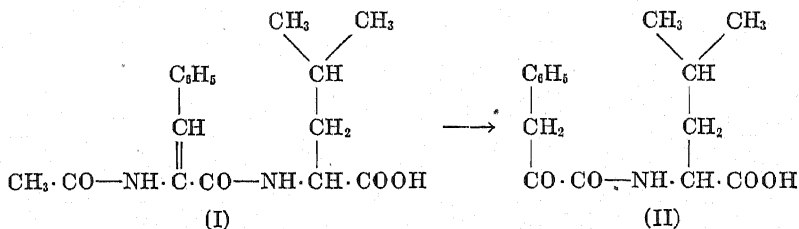
In this manner, phenylpyruvyl derivatives of glycine, *l*-leucine, *dl*-leucine, *l*-glutamic acid, and *l*-phenylalanine have been prepared. As has been noted in the case of phenylpyruvic acid (9), the phenylpyruvyl compounds tend to decompose when stored in solid form. The characterization of several of these compounds is difficult since they decompose during the melting point determination. The oximes, however, are stable and have satisfactory melting points.

In earlier experiments with pyruvylamino acids, it was found that pyruvyl-*dl*-phenylalanine is readily split by extracts of commercial pancreatin, whereas pyruvylglycine and pyruvyl-*dl*-alanine appeared to be resistant to the action of such enzyme extracts (10). The enzymatic

TABLE I

Action of Crystalline Carboxypeptidase on Phenylpyruvylamino Acids

Temperature 25°.

Substrate (0.05 M)	Enzyme concentration, protein N per cc. test solution	pH	Time	Hydrolysis
	mg.		*hrs.*	*per cent*
Phenylpyruvyl-*l*-phenylalanine	0.04	7.3	2	35
			4	43
Phenylpyruvyl-*l*-leucine	0.10	7.4	2	19
			4	28
Phenylpyruvyl-*l*-glutamic acid	0.40	7.4	24	10
			48	14
Phenylpyruvylglycine	0.40	7.3	24	2
			48	4
Carbobenzoxyglycyl-*l*-phenylalanine	0.0004	7.3	0.5	26
			1	42
Carbobenzoxyglycyl-*l*-phenylalanine + phenylpyruvic acid (0.05 M)	0.0004	7.3	0.5	17
			1	32

hydrolysis of the phenylalanine compound was attributed to the action of pancreatic carboxypeptidase. At that time, however, the crystalline carboxypeptidase of Anson (11) was not available. Accordingly, it appeared desirable to test the action of crystalline carboxypeptidase on phenylpyruvylamino acids. The data presented in Table I show that crystalline pancreatic carboxypeptidase can hydrolyze keto acylamino acids and that, of the compounds tested, phenylpyruvyl-*l*-phenylalanine is split most rapidly, phenylpyruvyl-*l*-leucine less rapidly, phenylpyruvyl-*l*-glutamic acid very slowly, and phenylpyruvylglycine slowest of all. These findings are in general accord with the data presented previously (12) on the effect of the nature of the terminal amino acid on the rate of peptide hydrolysis by carboxypeptidase. However, quantitative relations

could not be established since the hydrolysis of the phenylpyruvylamino acids does not follow satisfactory first order kinetics, in contrast to the behavior of the best available substrate for carboxypeptidase, carbobenzoxyglycyl-*l*-phenylalanine. This deviation from first order kinetics may be attributed to inhibition of the enzyme by phenylpyruvic acid, which is formed during hydrolytic cleavage. Similar inhibition has been observed (13) when chloroacetylamino acids are used as substrates for carboxypeptidase, and it has been demonstrated that chloroacetic acid is responsible for this effect.

EXPERIMENTAL

Phenylpyruvylglycine—20 gm. of acetyldehydrophenylalanylglycine (14) were heated under a reflux for 2 hours on the steam bath with a mixture of 35 cc. of glacial acetic acid and 40 cc. of N hydrochloric acid. The resulting clear solution was chilled overnight at 2°, whereupon crystals of phenylpyruvylglycine separated. This substance was recrystallized from methanol-water (1:2). Yield, 7.2 gm., m.p., 167–168°.

$C_{11}H_{11}O_4N$. Calculated. C 59.7, H 5.0, N 6.3
221.2   Found.   " 59.7, " 5.0, " 6.3

The oxime was prepared by treatment of 3.5 gm. of phenylpyruvylglycine with a solution of 2 gm. of hydroxylamine hydrochloride and 4 gm. of sodium acetate in 20 cc. of water. After a few minutes there resulted a clear solution from which crystals began to separate. The mixture was left at room temperature for 2 days and then acidified to Congo red with dilute hydrochloric acid. The oxime thus obtained weighed 3.7 gm. After recrystallization from methanol-water, the melting point was 141°.

$C_{11}H_{12}O_4N_2$. Calculated. C 55.9, H 5.1, N 11.9
236.2   Found.   " 55.7, " 5.2, " 11.6

Phenylpyruvyl-l-leucine—22 gm. of acetyldehydrophenylalanyl-*l*-leucine (15) were heated under a reflux for 1½ hours with a mixture of 60 cc. of glacial acetic acid and 60 cc. of 2 N hydrochloric acid. The resulting clear solution was chilled, whereupon the desired product crystallized. The crude material was recrystallized twice from methanol-water. Yield, 5.1 gm.

$C_{15}H_{19}O_4N \cdot H_2O$. Calculated. C 61.0, H 7.1, N 4.8
295.3   Found.   " 61.3, " 6.8, " 4.9

The oxime was prepared in a manner similar to that employed for phenylpyruvylglycine. From 1.5 gm. of the above compound, 1.2 gm.

of the oxime were obtained. After recrystallization from a mixture of methanol and dilute hydrochloric acid, the melting point was 165°.

$C_{15}H_{20}O_4N_2$.   Calculated.   C 61.6, H 6.9, N 9.6
292.3   Found.   " 61.3, " 6.9, " 9.6

Phenylpyruvyl-dl-leucine—27 gm. of acetyldehydrophenylalanyl-*dl*-leucine (prepared in the same manner as described for the *l* form) were refluxed for $1\frac{1}{2}$ hours with a mixture of 75 cc. of glacial acetic acid and 75 cc. of 3 N hydrochloric acid. The resulting clear solution was concentrated under reduced pressure to a volume of about 40 cc. The crystals which separated were recrystallized from methanol-water (2:1). Yield, 5.8 gm.

$C_{15}H_{19}O_4N$.   Calculated.   C 65.0, H 6.9, N 5.0
277.3   Found.   " 65.0, " 7.0, " 5.0

The oxime was prepared in the same manner as described for the *l* form. M.p., 158–159°.

$C_{15}H_{20}O_4N_2$.   Calculated.   C 61.6, H 6.9, N 9.6
292.3   Found.   " 61.9, " 7.0, " 9.7

Phenylpyruvyl-l-glutamic acid—15 gm. of acetyldehydrophenylalanyl-*l*-glutamic acid (8) were refluxed for $1\frac{1}{2}$ hours with 100 cc. of 2 N hydrochloric acid. The solution was concentrated under reduced pressure to a volume of about 20 cc. The resulting crystals were recrystallized from water. Yield, 4.7 gm., m.p., 142–143°.

$C_{14}H_{15}O_6N$.   Calculated.   C 57.3, H 5.2, N 4.8
293.3   Found.   " 57.4, " 5.2, " 4.8

Phenylpyruvyl-l-phenylalanine—A mixture of 22 gm. of acetyldehydrophenylalanyl-*l*-phenylalanine (14), 50 cc. of glacial acetic acid, and 50 cc. of 2 N hydrochloric acid was heated for 2 hours in a bath at 100°. The solution was chilled to 0°, and 75 cc. of water were added. The product which separated was recrystallized twice from methanol-water.

$C_{18}H_{17}O_4N$.   Calculated.   C 69.4, H 5.5, N 4.5
311.3   Found.   " 69.5, " 5.6, " 4.5

The oxime was prepared in a manner similar to that described for phenylpyruvylglycine. It melted at 160°.

$C_{18}H_{18}O_4N_2$.   Calculated.   C 66.1, H 5.5, N 8.6
326.3   Found.   " 66.3, " 5.7, " 8.7

Enzymatic Studies—The crystalline carboxypeptidase was prepared and recrystallized according to the directions of Anson (11). The procedure employed in the determination of enzymatic hydrolysis was the same as that described earlier (13).

SUMMARY

A method is described for the preparation of phenylpyruvylamino acids from the corresponding acetyldehydrophenylalanylamino acids. The phenylpyruvylamino acids are hydrolyzed by crystalline pancreatic carboxypeptidase.

BIBLIOGRAPHY

1. Linderstrøm-Lang, K., in Annual review of biochemistry, Stanford University, 8, 37 (1939).
2. Agren, G., *Acta physiol. Scand.*, 1, 233 (1940).
3. Herbst, R. M., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, 4, 75 (1944).
4. Bergmann, M., and Fruton, J. S., *Ann. New York Acad. Sc.*, 45, 409 (1944).
5. Herbst, R. M., and Shemin, D., *J. Biol. Chem.*, 147, 541 (1943).
6. Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, 187, 196 (1930).
7. Bergmann, M., Miekeley, A., and Kann, E., *Z. physiol. Chem.*, 146, 247 (1925).
8. Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, 449, 277 (1926).
9. Herbst, R. M., and Shemin, D., in Organic syntheses, New York, coll. 2, 519 (1943).
10. Bergmann, M., and Schleich, H., *Z. physiol. Chem.*, 207, 361 (1932).
11. Anson, M. L., *J. Gen. Physiol.*, 20, 663 (1937).
12. Stahmann, M., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 164, 753 (1946).
13. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, 134, 225 (1940).
14. Doherty, D. G., Tietzman, J. E., and Bergmann, M., *J. Biol. Chem.*, 147, 617 (1943).
15. Behrens, O. K., Doherty, D. G., and Bergmann, M., *J. Biol. Chem.*, 136, 61 (1940)

SOME OBSERVATIONS CONCERNING SPHINGOMYELIN AND SPHINGOMYELIN REINECKATE

By M. H. HACK

(*From the Department of Psychiatry, University of Illinois College of Medicine, Illinois Neuropsychiatric Institute, Chicago*)

The sphingomyelin content of whole brain has been estimated by isolation to be about 1 per cent of the dry weight (1, 2), whereas concentrations of the order of 5 per cent of the dry weight have been reported when suitable extracts of whole brain were assayed by the reineckate procedure (3) (*cf.* Table I). In the present work, both sphingomyelin and sphingomyelin reineckate were studied in order to determine the origin of these differences.

Materials and Methods

Sphingomyelin was prepared from whole beef brain[1] (Preparation S-10) by the method of Klenk and Rennkamp (10) and from whole beef and human brain (Preparations S-1 and H-S) by the method of Thannhauser and Setz (3). The cerebrosides kerasin and phrenosin were obtained from whole human brain by the method of Rosenheim (11). A cerebroside-sphingomyelin mixture (Preparation C-S) was prepared from the ethyl alcohol extract of acetone-dried beef brain. A sample containing a mixture of cephalins was obtained from human brain by the method of Folch (12). Three whole, frozen dried cat brains were separately worked up according to the procedure of Thannhauser and Setz (3) for the determination of sphingomyelin in tissue; the resulting precipitates are hereafter designated Preparations C-B-1-R, C-B-2-R, and C-B-3-R (R denotes reineckate). Preparation C-B-4 was a similar cat brain preparation except that the ammonium reineckate was omitted. All sphingomyelin reineckates here reported were prepared according to the directions of Thannhauser and Setz (3). The ammonium reineckate (13) contained the theoretical amount of nitrogen.

The above preparations were analyzed for the presence of the following according to the standard procedures: nitrogen by micro-Kjeldahl (14) and by micro-Dumas (15), phosphorus (16), chromium (17), glycerol (15), and choline (18). Hexose was detected by the method of Foulger (19). All values reported were the averages of duplicate determinations which

[1] A considerable quantity of acetone-dried beef brains was generously supplied by The Wilson Laboratories, Chicago.

agreed within the limits described for the method and all procedures were regularly checked with known amounts of standard substances.

TABLE I

Sphingomyelin Content of Whole Brain

Authority	Source	Per cent wet weight	Per cent dry weight	Method
Schuwirth (2)	Man (average of 2 normal adults)	0.18	0.90	Isolation
Thannhauser *et al.* (4)	Man		4.51 6.82	Reineckate (gravimetric)
Erickson *et al.* (5)	Dog		4.00 6.00	Reineckate*
Kaucher *et al.* (6)	Beef		4.95	"
Hunter (7)	Cat (average of 9 animals)	1.25		"
Taylor (8)	Cat (average of 10 animals)	1.15	4.62	"
Williams *et al.* (9)	Rat (age 15 days) " (" 45 ") " (" 70 ")		3.93 3.99 4.27	"
Hack, this paper Whole brain, each worked up as a single sample	Man (normal adult)	0.23	1.37	Isolation
	Cat (Preparation C-B-4)	0.19	0.86	"
	Cat (Preparation C-B-1)	0.99	4.47	Reineckate*
	Cat (Preparation C-B-2	1.15	6.05	"
	Cat (Preparation C-B-3)	0.83†	4.00	"

* Based on P content.

† Accident resulting in some loss of material; the value is somewhat low.

Results

The isolation of pure lipide components is laborious; consequently the values of sphingomyelin obtained by isolation may be low.

The analyses of the sphingomyelins are shown in Table II. The following observations were also made. Analysis of Preparation S-1 for nitrogen by the micro-Kjeldahl method showed values that ranged from 2.70 per cent when digested for 15 minutes to 3.19 per cent when digested for 120 minutes. These values are substantially lower than those obtained by combustion analysis (3.76 per cent). Similar low Kjeldahl nitrogen values were observed for all of the lipide samples here described which contained choline. It is generally recognized that the estimation of nitrogen in

choline by the Kjeldahl procedure requires drastic conditions of digestion. Since, theoretically, one-half of the nitrogen of sphingomyelin is in the choline, low yields were expected. The values of nitrogen obtained by combustion reported here for these sphingomyelins are somewhat higher than Kjeldahl values reported elsewhere (*cf.* Table III). Also, analysis of sphingomyelin for choline yielded low values as follows: hydrolysis of Preparation S-1 with 0.5 N barium hydroxide at 100° for 3, 6, and 24 hours gave 8.3, 11.5, and 12.2 per cent choline, respectively. The theoretical choline content of the sphingomyelin ranges from 14.5 to 16.8 per cent. These low values for choline were not due to loss, since similar treatment of choline chloride with barium hydroxide for 24 hours did not prevent

TABLE II

Analysis of Lipide Preparations

Preparation	P:N:Cr	P	N (Dumas)	Cr	Glycerol	Hexose
		per cent	*per cent*	*per cent*	*per cent*	
S-10	1:2.19	3.88	3.85		0	0
S-10-R	1:5.70:1.19	2.75	7.08	5.50	0	0
S-1	1:2.20	3.77	3.76		0.63	0
S-1-R	1:5.38:0.53	3.10	7.55	2.78	0.53	0
H-S	1:1.96	3.82	3.38			0
H-S-R	1:5.40:0.75	3.04	7.44	3.82		0
C-S	1:5.66	0.94	2.40		1.23	+
C-S-R	1:11.90:0.58	0.65	3.51	0.63	0.86	+
C-B-1-R	1:6.82:0.61	1.42	4.37	1.45	2.68	+
C-B-2-R	1:7.14:0.64	1.38	4.45	1.48	2.60	+
C-B-3-R	1:7.55:0.68	1.42	4.85	1.62	3.04	+
C-B-4	1:9.52	0.50	2.15		1.89	+

recovery of 96 per cent of it in control experiments. Hydrolysis of Preparation S-1 with 6 N hydrogen chloride in methyl alcohol (24) yielded low and variable results. Since the choline of sphingomyelin is presumed to be linked to phosphoric acid as phosphorylcholine, the low yields of choline are in keeping with the studies of Baer and McArthur (25) who have shown that phosphorylcholine is extremely resistant to both acid and alkaline hydrolysis. Hence choline determinations on lipide extracts containing sphingomyelin must be interpreted with caution. Thus, if the lecithin content of tissue is calculated from the total choline less the choline equivalent of the sphingomyelin found to be present, the values obtained for lecithin would be low.

Based on this resistance to hydrolysis, a new method for the determination of sphingomyelin was recently proposed by Schmidt *et al.* (26). "The method is based on the observation that lecithin, hydrolecithin

and all cephalins are quantitatively transformed into acid-soluble P compounds during incubation with aqueous N potassium hydroxide for 15 hours at 37°. Under these conditions the P-containing group of sphingomyelin remains insoluble in acids." This observation has been substantiated with both Preparations S-1 and S-10. Cephalin (containing 3.52 per cent phosphorus) when treated for 16 hours with N potassium hydroxide at 37° yielded 3.30 per cent phosphorus.

Since the determination of sphingomyelin as the reineckate is generally carried out on crude extracts, it seems reasonable to expect that, unless the reine kate procedure is specific for sphingomyelin, the resulting precipitate will be a mixture of lipides. Reference to Table II shows that

TABLE III

Analysis of Brain Sphingomyelins

Authority	P:N	Phosphorus	Nitrogen	N method
		per cent	*per cent*	
Thudichum (20)	1:2.02	3.24	2.96	?
Levene (21)	1:2.08	3.99	3.78	Kjeldahl
Merz (22)	1:1.95	4.10	3.63	"
	1:1.96	4.03	3.57	"
	1:1.93	4.09	3.56	"
	1:1.99	3.90	3.50	"
	1:1.91	3.96	3.42	"
	1:1.95	4.01	3.53	"
Tropp and Eckardt (23)	1:2.05	3.72	3.45	Dumas
Taylor (8)	1:1.95	3.78	3.30	?
Klenk and Rennkamp (10)	1:2.02	3.89	3.57	Kjeldahl
Hack, this paper	1:2.19	3.88	3.85	Dumas

the reineckates prepared from those samples that contained glycerol and hexose also contained them, whereas sphingomyelin contains neither.

The presence of glycerol may be accounted for as follows: 12.421 mg. of the methanol-soluble fraction of cephalin (1.50 per cent P) yielded 7.802 mg. of a lavender-gray precipitate (0.75 per cent P) when assayed for sphingomyelin as the reineckate. A mixture containing 19.508 mg. of the above cephalin fraction and 15.347 mg. of Preparation S-10 yielded 30.893 mg. of a lavender-gray precipitate which contained 1.80 per cent P and 1.13 per cent glycerol. Similarly 6.212 mg. of Preparation S-10 and 23.319 mg. of the cephalin fraction yielded 25.580 mg. of a lavender-gray precipitate which contained 1.42 per cent P and 1.50 per cent glycerol.

The presence of hexose was accounted for in a similar manner. Reineckates were prepared from 16.248 mg. of pure phrenosin (0.00 per cent P, 1.70 per cent N (Kjeldahl); theory 1.69 per cent N) and 22.956 mg. of

pure kerasin (0.00 per cent P, 1.79 per cent N (Kjeldahl); theory 1.73 per cent N). The reineckates weighed 14.040 and 18.022 mg. respectively and were of the typical pink color. Therefore, sphingomyelin determined as the reineckate can yield high values when measured either on the basis of its P content (5) or gravimetrically (3, 24).

The following experiments were performed in an attempt to explain the mechanism of the formation of sphingomyelin reineckate. Since the procedure involves the acidification of a hot concentrated methyl alcoholic solution of sphingomyelin to which is added a saturated solution of ammonium reineckate in methyl alcohol, which is then refrigerated at 0° for several hours, it was thought pertinent to examine the properties of

TABLE IV

Calculated Theoretical Elementary Composition of Sphingomyelins and Sphingomyelin Reineckates (Based on Monomeric Conception)

	P:N:Cr	P	N	Cr
		per cent	*per cent*	*per cent*
Stearyl sphingomyelin	1:2	4.15	3.74	
" " reineckate..........	1:8:1	2.90	10.4	4.86
Lignoceryl "	1:2	3.73	3.36	
" " reineckate..........	1:8:1	2.68	9.70	4.50
Nervonyl "	1:2	3.74	3.37	
" " reineckate..........	1:8:1	2.68	9.72	4.52
Palmityl "	1:2	4.30	3.88	
" " reineckate.....	1:8:1	2.98	10.7	4.98

the reagents under such conditions. 3 mg. of Preparation S-1 were soluble in 1 ml. of neutral methyl alcohol at 0°, while less than 1 mg. per ml. was soluble at the same temperature in methyl alcohol containing 1 drop of concentrated hydrochloric acid per ml. Similar solubilities were found to be true also of Preparations S-10, H-S, C-S, and C-B-4. The saturated methyl alcoholic solution of ammonium reineckate contained 220 mg. per ml. at 25°. On refrigeration a few small crystals formed, but on acidification with 1 drop of concentrated hydrochloric acid per ml. at room temperature there was a copious crystalline precipitate which increased on refrigeration. It would, therefore, seem that the precipitation of sphingomyelin under the conditions described (3) for the preparation of the reineckate might occur without the formation of a salt. Neither reineckates prepared from the sphingomyelin nor those from crude extracts on analysis show values (*cf.* Table II) indicating a stoichiometric relationship between sphingomyelin and Reinecke acid (ammonium reineckate + hydrogen chloride = Reinecke acid + ammonium chloride) (*cf.* Table IV).

Instead, it would seem reasonable to consider sphingomyelin reineckate as a mixture produced by adsorption and salted-out.

This hypothesis is substantiated by the following experiments. When 50 mg. samples of Preparations S-1-R, C-S-R, C-B-1-R, C-B-2-R, and C-B-3-R were extracted with acetone in a Soxhlet apparatus, complete extraction occurred within 24 hours, resulting in a pink extract which when cooled to 25° yielded in all cases a white precipitate, presumably sphingomyelin, or cerebroside, or both. After only 6 hours extraction 50 mg. samples of Preparations C-B-1-R, C-B-2-R, and C-B-3-R lost 67, 63, and 64 per cent respectively to the extracts. These extracts were dried *in vacuo;* the residues contained 1.45, 1.45, and 1.65 per cent phosphorus respectively. Similarly 6 hours extraction of Preparation S-1-R resulted in a loss of only 37 per cent to the extract which, dried *in vacuo,* contained 2.16 per cent phosphorus. In contrast, 6 hours extraction of Preparation S-1 showed a loss of only 2 per cent to the extract, which dried *in vacuo* contained 1.78 per cent phosphorus. Reinecke acid was found to be very soluble in acetone, even at 0°. All of the above lipide preparations were found to be very soluble in chloroform, even at 0°, whereas neither Reinecke acid nor any of the sphingomyelin reineckates investigated were soluble in chloroform at 0°. These results suggest that the Reinecke acid and sphingomyelin were adsorbed on one another and that solvent action was sufficient to disturb the resulting complex, thus permitting a separation of the two components. The difference in solubility between Preparations C-B-1-R, etc., and S-1-R was presumably due to the presence of lipides other than sphingomyelin, as shown above.

If formation of the reineckate were an adequate method of assay, then reasonable recoveries of pure sphingomyelin should be possible. According to Klenk and Rennkamp (10) only Preparation S-10 of this series can be considered pure sphingomyelin. However, both Preparations S-1 and H-S would have been considered pure at the time the reineckate method was proposed.

The actual recoveries were as follows:

Preparation		Preparation	Recovered	
	mg.		*mg.*	*per cent*
S-10	23.375	S-10-R	28.370	= 86
S-1	30.730	S-1-R	29.922	= 80
H-S	26.665	H-S-R	25.459	= 76

The difference in total phosphorus (*cf.* Table II) between Preparations H-S and H-S-R was 0.222 mg. 0.217 mg. of phosphorus was recovered in the combined filtrate and washings, of which 0.127 mg. was accounted for in the filtrate. Similarly Preparation S-1 lost 0.230 mg. of phosphorus and Preparation S-10 lost 0.128 mg. Analysis of the ammonium reineckate

showed no phosphorus. Choline was demonstrated as the enneaiodide after hydrolysis of an aliquot of the combined filtrate and washings.

Reference to Table I shows that there is general agreement within each of the two methods, but it would seem that those values obtained by isolation are too low and those as the reineckate too high. The presence of glycerol in these preparations implies the presence of other phospholipides, and hexose, the presence of cerebrosides. The existing methods of lipide analysis do not permit a more reliable assay of sphingomyelin.

SUMMARY

1. Sphingomyelin and sphingomyelin reineckates have been prepared from several sources by standard procedures and subjected to chemical analysis.

2. Kjeldahl nitrogen values were found lower than Dumas nitrogen values, presumably due to choline.

3. Assay of sphingomyelin for choline yielded low values, presumably due to the resistance of phosphorylcholine to hydrolysis.

4. Reineckates prepared from crude extracts contained both glycerol and hexose, implying therefore the presence of lipides other than sphingomyelin.

5. The methanol-soluble fraction of cephalin and the cerebrosides kerasin and phrenosin formed reineckates.

6. Recovery of pure sphingomyelins as the reineckate were low.

7. The behavior of a saturated solution of ammonium reineckate in the presence of HCl at 0° and the solubility of sphingomyelin in acid alcohol suggest the formation of an adsorption complex, which was confirmed by leaching.

BIBLIOGRAPHY

1. Klenk, E., Z. physiol. Chem., **262,** 128 (1939).
2. Schuwirth, K., Z. physiol. Chem., **263,** 25 (1940).
3. Thannhauser, S. J., and Setz, P., J. Biol. Chem., **116,** 527, 533 (1936).
4. Thannhauser, S. J., Benotti, J., Walcott, A., and Reinstein, H., J. Biol. Chem., **129,** 717 (1939).
5. Erickson, B. N., Avrin, I., Teague, D. M., and Williams, H. H., J. Biol. Chem., **135,** 671 (1940).
6. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., Arch. Biochem., **3,** 203 (1943).
7. Hunter, F. E., J. Biol. Chem., **144,** 439 (1942).
8. Taylor, J. D., Thesis, University of Rochester (1940).
9. Williams, H. H., Galbraith, H., Kaucher, M., Moyer, E. Z., Richards, A. J., and Macy, I. G., J. Biol. Chem., **161,** 475 (1945).
10. Klenk, E., and Rennkamp, F., Z. physiol. Chem., **267,** 145 (1941).
11. Rosenheim, O., Biochem. J., **8,** 110 (1914).
12. Folch, J., J. Biol. Chem., **146,** 35 (1942).

13. Blatt, A. H., in Organic syntheses, New York, coll. **2** (1943).
14. Ma, T. S., and Zuizaga, G., *Ind. and Eng. Chem., Anal. Ed.*, **14,** 280 (1942).
15. Roth, H., Quantitative organic microanalysis of Fritz Pregl, translated by Daw, E. B., Philadelphia, 3rd edition (1937).
16. Horecker, B. L., Ma, T. S., and Haas, E., *J. Biol. Chem.*, **136,** 775 (1940).
17. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, **147,** 363 (1943).
18. Entenman, C., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **155,** 13 (1944).
19. Foulger, J. H., *J. Biol. Chem.*, **99,** 207 (1932).
20. Thudichum, J. L. W., Die chemische Konstitution des Gehirns des Menschen und der Tiere, Tübingen (1901).
21. Levene, P. A., *J. Biol. Chem.*, **24,** 69 (1916).
22. Merz, W., *Z. physiol. Chem.*, **193,** 59 (1930).
23. Tropp, C., and Eckardt, B., *Z. physiol. Chem.*, **245,** 163 (1937).
24. Thannhauser, S. J., Benotti, J., and Reinstein, H., *J. Biol. Chem.*, **129,** 709 (1939).
25. Baer, E., and McArthur, C. S., *J. Biol. Chem.*, **154,** 451 (1944).
26. Schmidt, G., Benotti, J., and Thannhauser, S. J., *Federation Proc.*, **5,** 152 (1946)

A SIMPLE QUALITATIVE TEST TO DISTINGUISH BETWEEN PROTOPORPHYRIN IX OR ITS ESTERS AND PORPHYRINS CONTAINING NO VINYL GROUP*

By EDITH JU-HWA CHU†

(From the Department of Medicine, University of Minnesota Hospital, Minneapolis)

Since a satisfactory procedure of preparing pure protoporphyrin IX dimethyl ester from hemoglobin (1) has been worked out, it is interesting to investigate direct reduction of protoporphyrin IX dimethyl ester into mesoporphyrin IX dimethyl ester. Although these compounds, the melting points of which are too close to be of use for their differentiation, can be distinguished by HCl number (2) or absorption spectra, the simple and rapid qualitative test here described has proved useful in the above investigation.

Protoporphyrin IX dimethyl ester used in this experiment was prepared by a simplified modification of the procedure (1) previously described. A sample of 5 ml. of red blood cells was refluxed with 100 ml. of 10 per cent oxalic acid in acetone for half an hour. The reaction mixture was filtered and washed with a little acetone. The colorless residue was discarded and the solution was then worked up by the procedure outlined in the previous paper. When in place of the 10 per cent oxalic acid solution 5 per cent and 1 per cent solutions of oxalic acid in acetone were tried, the yields of protoporphyrin IX dimethyl ester were 5 mg. and 3 mg. respectively per ml. of red blood cells.

A procedure for preparing protoporphyrin IX dimethyl ester recently described by Grinstein and Camponovo (3) was also tried and modified. A sample of 5 ml. of red blood cells was stirred with 100 ml. of 1 per cent oxalic acid in methanol. The dark solution separated from the residue by centrifuging was shaken with 5 gm. of stannous chloride and mixed with an equal volume of methanol previously saturated with hydrogen chloride gas. The reaction mixture was allowed to stand at room temperature for 1 to 2 hours and was worked up as usual. By this modified method a pure product was obtained without the necessity of chromatographic purification, the yield being 2.8 mg. per ml. of red blood cells.

Mesoporphyrin IX dimethyl ester was also directly prepared from red blood cells. The acetone solution resulting from refluxing 5 ml. of red

blood cells with 100 ml. of 1 per cent oxalic acid in acetone was distilled under reduced pressure at room temperature to remove the solvent. The residue was mixed with excess water and centrifuged. The dark residue was washed once with water and dried on a steam bath. It was then reduced with a mixture of 0.35 ml. of hydriodic acid (sp. gr. 1.7) and 2.6 ml. of glacial acetic acid according to the procedure of Fischer and Kögl (4). A methanol solution of crude mesoporphyrin thus obtained was mixed with an equal volume of methanol previously saturated with hydrogen chloride and allowed to stand at room temperature for $1\frac{1}{2}$ hours. The ester was extracted as usual and purified by chromatographic separation, the yield being 3 mg. per ml. of red blood cells.

For the qualitative test protoporphyrin IX dimethyl ester and mesoporphyrin IX dimethyl ester were respectively dissolved in chloroform as pink solutions. Then to each tube were added 3 drops of concentrated hydrochloric acid with vigorous shaking. Changes in color were observed in transmitted daylight. The solution containing mesoporphyrin IX ester is purple, while that containing protoporphyrin IX ester shows a green shade. In both tubes dihydrochlorides were formed which showed different colors toward transmitted daylight. In either tube the pink color was restored by addition of excess saturated sodium acetate solution and the test could be repeated. Free protoporphyrin IX gave the same color change as its ester, while free mesoporphyrin and other porphyrins, such as coproporphyrin, containing no vinyl group behaved toward hydrochloric acid just like mesoporphyrin IX dimethyl ester. Therefore this simple test serves as a rapid means of distinguishing between porphyrins of the protoporphyrin type and porphyrins containing no vinyl group. The test is sensitive in a concentration of 10 to 100 γ per ml. of testing solution.

SUMMARY

Protoporphyrin IX dimethyl ester and mesoporphyrin IX dimethyl ester were respectively prepared by modified procedures. A simple color test to distinguish between these two porphyrins is described.

BIBLIOGRAPHY

1. Chu, E. J.-H., *J. Biol. Chem.*, **163**, 713 (1946).
2. Treibs, A., *Ann. Chem.*, **476**, 4 (1929).
3. Grinstein, M., and Camponovo, P. B., *Rev. Soc. argent. biol.*, **21**, 301 (1945).
4. Fischer, H., and Kögl, F., *Z. physiol. Chem.*, **138**, 262 (1924).

PRODUCTION AND PURIFICATION OF PENICILLINASE

By G. A. LePAGE, J. F. MORGAN, and M. E. CAMPBELL

(*From the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada*)

The ability of various bacterial species to inactivate penicillin was first observed by Abraham and Chain (1) in 1940. The active agent was extracted from crushed cells of *Escherichia coli*, its enzymatic nature determined, and the name "penicillinase" proposed.

Harper (2), Smith and Smith (3), and Proom (4) have reported methods for the extraction of penicillinase from the cells and culture filtrates of paracolon organisms, while Duthie (5) has prepared an extracellular penicillinase from *Bacillus subtilis*. Benedict, Schmidt, and Coghill (6) surveyed 65 bacterial species for antipenicillin activity, and obtained the highest yield of crude enzyme from an unclassified spore former closely related to the *Bacillus cereus* group. McQuarrie and Liebmann (7) have also reported the preparation of impure penicillinase from unidentified Gram-negative rods but do not report the yields obtained.

Despite the many publications, isolation of penicillinase in highly active form has not previously been reported. The importance of this enzyme in the sterility testing of penicillin, and possibly in penicillin assay (8), prompted this investigation.

EXPERIMENTAL

A survey was made of penicillinase-producing bacteria, including paracolon and coliform cultures and various organisms of the Bacillaceae. The latter group included contaminants from penicillin assay plates, spore formers isolated from clarase, NRRL 569,[1] and strains of *Bacillus cereus*[2] and *Bacillus megatherium*.[2] Cellular material was investigated by the method of Harper (2), the culture filtrates by assay as described in this paper.

Comparative tests were conducted with all these organisms on the following media: Difco nutrient broth, peptone, yeast extract, yeast extract plus peptone, yeast extract plus glucose, and peptone plus glucose. Addition of glucose to media tended to inhibit enzyme production. Aera-

---

[1] Obtained through the courtesy of Dr. R. G. Benedict, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois.

[2] Obtained through the courtesy of Mr. Clark Blackwood, Division of Applied Biology, National Research Council, Ottawa, Canada.

tion caused increase in cellular material but did not significantly modify the activity per individual cell.

Filtering the cultures through a Seitz filter was observed to remove most of the penicillinase activity.

One strain of *Bacillus megatherium* and the NRRL 569 culture were selected as the best enzyme producers. With three pH levels, 7.0, 7.5, and 8.0, it was shown that these cultures gave optimal production at neutrality.

Method of Assay—For test purposes the material to be assayed is diluted in sodium phosphate buffer, pH 7.0, to a concentration of 1 mg. per ml., and 4 ml. of solution were placed in a small tube. (In the case of culture fluids no previous dilution is required.) From this first tube 2 ml. are removed and halving dilutions made in buffer throughout a series. To each tube 0.2 ml. of a 10 unit per ml. solution of standard penicillin is added. After incubation for 1 hour at 37° a cup-plate assay is carried out, by an adaptation of the method of Schmidt and Moyer (9). Duplicate cups are filled with aliquots of the solution from each tube. Selecting three values or zone sizes with three adjacent halving dilutions did not always give a straight line response. Differences in response to penicillin occur with preparations in different stages of purification, making it difficult to assign a true value to any one preparation. Although the rate of diffusion of penicillin is influenced by various salts, and salt concentration, the high dilutions employed in the present experiments make this factor of little significance in assay. The dilution causing destruction of approximately 50 per cent of the penicillin was found to be the most satisfactory end-point. For convenience in comparing results this end-point has been called a "dilution unit."

It was observed that if a solution of standard penicillin several days old (but still assaying at 100 per cent potency by plate assay) was employed unaccountably high activities might be indicated for some enzyme preparations. Further, it appeared that pure crystalline sodium penicillin G was more rapidly destroyed than a 370 unit per mg. calcium penicillin G standard (supplied by the Food and Drug Administration, Washington).

The problem of inactivation of the enzyme has not yet been satisfactorily solved. For this reason values attached to different preparations are largely relative.

Media and Enzyme Adaptation—In selecting the best medium for enzyme production, combinations of inorganic ammonium salts, glucose, and minimal amounts of yeast extract were used in addition to the regular experimental media. All were suitably buffered to maintain neutrality during the growth period. A concentration of 0.15 per cent yeast extract

buffered in 0.2 M phosphate gave excellent production, but maximal yields were obtained when the yeast extract concentration was raised to 0.5 per cent.

With both organisms, and in all culture media, centrifugation of the cells removed about half the activity. Nevertheless enzyme yields were so large extracellularly that inclusion of cellular material was of no great benefit. In yeast extract medium, culture NRRL 569 appeared to produce about 8 times as much enzyme as *Bacillus megatherium* and was therefore selected for large scale penicillinase production.

With the addition of penicillin to the cultures during growth this choice of organism was fully justified. Cells of both cultures harvested after a 4 day growth period and treated by Harper's method (2) revealed that in the case of *Bacillus megatherium* addition of the penicillin substrate did not appreciably change the activity of the cells, while with NRRL 569 at least a 40-fold increase was observed. The extracellular enzyme produced was adaptive in the case of both organisms, although adaptation was more evident with NRRL 569.

From these experiments, the following method of production has evolved.

After two transfers in tubes of 0.5 per cent yeast extract buffered to pH 7.0 with 0.05 M phosphate, a 2 per cent inoculum (4 hour growth) is used to seed a carboy partially filled with yeast extract medium. Rapid aeration at 22–26° is maintained for 4 hours, at which point penicillin in distilled water (one-twentieth volume of total culture fluid) is added to give a final concentration in the carboy of 1 unit per ml. All additions of penicillin are made gradually through a partially closed rubber tubing connected aseptically to a capillary pipette. At the end of 24 hours a second addition of penicillin, in the same volume as previously, is made in 0.2 M phosphate buffer, pH 7.0, to a final concentration of 5 units per ml. At 48 hours a third penicillin dilution in 0.2 M buffer is prepared. One-half of this preparation is added gradually over a period of 6 hours and the remainder is added in one step at the end of this time period. A final concentration of 50 units per ml. is attained. After 96 hours growth is stopped. With this method of production, assay of the cell-free culture fluid for enzyme has given dilution unitage as high as 1:1,000,000.

If at any time during the growth period cell lysis is observed, no penicillin should be added for 24 hours. It is usual to observe with lysed cultures that the enzyme titer drops but that this drop is attended by a sharp rise in 24 hours. Because the pH, substrate addition, and amount of nutrient available to the cells are important, and a delicate balance must be maintained, a sterile sampling device is attached to the carboy through which a sample may be removed at any time during the growth period and ap-

propriate adjustments made as required. In particular, variation in both growth and enzyme production was observed with different lots of Difco yeast extract.

Isolation of Enzyme

Adsorption—The 4 or 5 day culture is centrifuged, in a Sharples super-centrifuge, and the cell paste discarded. The cell-free filtrate is cooled to 5° and Hyflo Super-Cel added to a concentration of 5.0 per cent. The mixture is stirred mechanically for 30 minutes in the cold. At the end of this time the Hyflo Super-Cel is filtered off with the aid of suction on Büchner funnels containing two layers of filter paper (No. 1, Whatman) and the enzyme-free filtrate rejected.

The pH of the culture filtrate at the end of the growth period is approximately 7.3. Maximal adsorption is obtained at pH of 6.5, decreased adsorption occurring at pH values above and below this figure. To avoid such loss of enzyme the culture filtrate is adjusted to pH 6.5 with glacial acetic acid before adsorption is begun.

Various adsorbing agents were tested in the penicillinase cultures and Hyflo Super-Cel was found most satisfactory because of completeness of adsorption and ease of elution.

Preliminary experiments showed that adsorption periods up to 20 minutes resulted in losses of non-adsorbed enzyme, totaling approximately 10 per cent of the original activity. Increasing the adsorption time to 30 minutes decreased these losses to 4 per cent. A second adsorption period with fresh Super-Cel failed to reduce materially this 4 per cent loss.

Elution—A critical factor in the isolation of penicillinase was the method of recovery from the adsorbing agent. Therefore, methods of elution were investigated in detail. The concentrated phosphate mixture of Woodruff and Foster (10) was tested together with a dilute buffer of the same proportionate composition. The suitability of ammonia water as an eluting agent was also studied over a concentration range of 0.015 to 0.15 M ammonium hydroxide (0.1 to 1.0 per cent aqueous dilution of concentrated ammonia water).

1 liter quantities of cell-free culture filtrate, pH 6.5, were stirred in the cold for 30 minutes with 50 gm. of Hyflo Super-Cel, and the adsorbing agent collected by filtration. Each lot of Super-Cel was suspended in 500 ml. of test solution with mechanical stirring, and elution carried out in the cold for 30 minutes. The Super-Cel was recovered by filtration, and the elution process repeated twice with volumes of 250 ml. of test fluid, elution periods of 30 and 20 minutes being employed. Aliquots of the eluates at each step were lyophilized and tested for penicillinase activity. The three eluates from each treatment were then combined and

a further aliquot removed for testing as a check measure. Results of this experiment are presented in Table I.

From these results, it is clear that of the several eluting agents tested 0.5 per cent ammonia water is most satisfactory. Above this concentration some decrease in eluted enzyme is observed, while below this level very little penicillinase is recovered. In accord with the findings of Woodruff and Foster (10) concentrated buffer solution was observed to be a fairly effective eluting agent, while diluted phosphate buffer was relatively ineffective. A solution of 30 per cent ammonium phosphate, pH 7.8, was as effective as the concentrated sodium phosphate buffer.

TABLE I

Comparative Recoveries of Penicillinase from Hyflo Super-Cel with Various Eluting Agents

Results expressed as dilution units multiplied by the dry weight of the eluates (mg.).

Eluting agent	1st eluate	2nd eluate	3rd eluate	Sum of eluates
M/15 phosphate buffer, pH 7.0	25,700	16,000	2,700	44,400
M/1.5 " " " 7.1	563,200	264,700	19,300	847,200
0.015 M ammonium hydroxide (0.1 %), pH 10.3	114,000	114,900	89,600	318,500
0.075 M ammonium hydroxide (0.5 %), pH 10.6	2,058,200	499,700	675,800	3,233,700
0.15 M ammonium hydroxide (1.0 %), pH 10.9	2,276,400	403,400	46,600	2,726,400

Although M/1.5 phosphate buffer appeared reasonably efficient, the total penicillinase recovered amounted to only 25 per cent of that removed by elution with 0.5 per cent ammonia water. The small proportion by weight of enzyme in this concentrated buffer solution and the prolonged period of dialysis necessary at a later stage in purification make this eluting agent generally unsuitable.

With the eluting fluids tested the enzyme recovered is present in the first two eluates. An important exception is to be noted with 0.5 per cent ammonia water, in which case very high values were obtained in the third eluate, although the actual weight of enzyme removed was small. The possibility that this may be of practical significance as a purification method is under consideration.

The method finally adopted consists of three separate elutions with 0.5 per cent ammonia water for periods of 30, 30, and 20 minutes.

Purification—The ammonia water eluate is made up to 50 per cent saturation with solid ammonium sulfate (35 gm. per 100 ml.) and the

solution is held in the cold for 2 to 3 hours until flocculation is complete. The precipitate is removed by centrifuging at 2000 R.P.M. for 60 minutes, the supernatant decanted, and the residue of inactive protein discarded. Considerable purification of the final product is effected at this step, since approximately 50 per cent of the total protein in solution is removed. Exhaustive tests have established that the loss of penicillinase at this step is negligible, representing only 0.2 to 0.7 per cent of the total original activity.

The 50 per cent ammonium sulfate supernatant is made up to 100 per cent saturation and held overnight in the cold. The tiny floccules which form are removed by passage through a fritted glass filter of F porosity. The enzyme is removed from the surface of the filter, suspended in distilled water, transferred to a cellophane sac, and dialyzed against distilled water in the cold for a period of 24 to 48 hours. The enzyme is finally lyophilized and preserved in the cold. The final product obtained is a grayish brown in color and of a light, flaky or feathery consistency.

Loss of enzyme during the final filtration is negligible, provided a fritted filter of porosity F is employed. Repeated tests have failed to demonstrate loss of penicillinase activity during dialysis, as observed by Woodruff and Foster (10) and by Duthie (5), although in some instances dialysis was deliberately prolonged for 96 hours.

Attempts to purify the enzyme by precipitation with acetone or alcohol proved unsatisfactory, since almost complete loss of activity resulted.

Final yields, activity of the final product, and amount of inactive protein are summarized in Table II.

On the basis of both weight and activity of the final product, the enzyme obtained by 0.5 per cent ammonia water elution is far superior to that prepared by other methods. A considerably less active enzyme is obtained with 1.0 per cent ammonia water, while a concentration of 0.1 per cent is completely unsatisfactory. Repeated preparations employing 0.6 per cent ammonia water have resulted in enzymic material with a yield and activity comparable to that obtained at the 0.5 per cent level. It would appear that concentrations of ammonia water from 0.5 to 0.6 per cent are required for optimal recoveries of penicillinase.

It is of interest to note that as the concentration of ammonia water in the eluting fluid is increased from 0.1 to 1.0 per cent the weight of inactive protein decreases. The yield of active material is greatest at 0.5 per cent.

Comparison of the total activity of the final enzyme, Table II, with the total activity present in the eluates, Table I, shows complete lack of agreement. As yet, no satisfactory explanation for these divergent results can be offered.

Activity—Solution of the purified penicillinase was best accomplished

in 0.1 or 0.5 per cent ammonia water, since the material was largely insoluble in distilled water, sodium chloride solutions, or neutral phosphate buffers. Once dissolved in dilute ammonia water, the enzyme did not precipitate when added to penicillin solutions made up in phosphate buffer at neutrality.

It was observed that destruction of penicillin by this enzyme sharply declined in unbuffered media with an accompanying drop in pH. 0.05 M sodium phosphate buffer, pH 7.0, proved unable to prevent this fall in pH, buffer of 0.2 M strength being required. These results would indicate

TABLE II

*Yields and Relative Activity of Penicillinase Prepared by Representative Elution Methods and Ammonium Sulfate Fractionation**

Elution fluid	Inactive protein†	Enzyme‡	Activity	Total activity
	mg.	*mg.*	*dilution units per mg.*	*dilution units*
M/15 phosphate buffer, pH 7.0	23.2	52.5	2,000	105,000
M/1.5 " " " 7.1	24.7	34.3	525,000	18,007,500
0.015 M ammonium hydroxide (0.1 %), pH 10.3	50.3	45.2	65,500	2,960,600
0.075 M ammonium hydroxide (0.5 %), pH 10.6	40.3	56.7	2,100,000	119,070,000
0.15 M ammonium hydroxide (1.0 %), pH 10.9	36.2	53.3	525,000	27,982,500

* All yields reported are based on 1 liter of cell-free culture filtrate.

† Inactive protein removed at 50 per cent saturation with ammonium sulfate. The values represent dry weight after dialysis.

‡ Enzyme precipitated at 100 per cent saturation with ammonium sulfate. The values are expressed as dry weight after dialysis.

that penicillinase possesses a rather sharp optimal pH for enzymic activity. The pH curve will be given in a later publication.

In studying the action of this enzyme, solutions of commercial penicillin of 100,000 units per ml. were prepared in 0.2 M phosphate buffer. The enzyme was dissolved in 0.1 or 0.5 per cent ammonia water to a concentration of 1.0 mg. per ml. The reaction was allowed to proceed at 25° for selected periods of time. Appropriate dilutions were then made and tested for residual penicillin.

Within the limitations of this test, 1.0 mg. of the purified enzyme completely destroyed 100,000 units of penicillin within 10 minutes. Further investigation of the assay method with purified enzyme is required before any definite unitage of penicillinase can be established.

Sterility—Attempts to sterilize penicillinase solutions by Seitz filtration

proved unprofitable, since large amounts of enzyme were bound by the asbestos pads. Sterilization was achieved by passage of the enzyme through a fritted glass filter of UF porosity. It was found most convenient to dissolve the enzyme in 0.1 per cent ammonia water to a concentration of 2.0 mg. per ml. After filtration of this solution, an equal volume of 0.1 per cent ammonia water was passed through the filter. By this method a sterile 1.0 mg. per ml. penicillinase solution was obtained, with negligible loss of activity during the filtration.

The application of this highly purified penicillinase to the routine sterility testing of penicillin and the optimal conditions for penicillinase activity are under investigation.

SUMMARY

The production of penicillinase by representative organisms has been studied in a variety of test media. Greatest production of the enzyme was obtained with culture NRRL 569 grown with aeration for 96 hours in buffered yeast extract broth. The enzyme has been shown to be extracellular and adaptive in nature.

Isolation of penicillinase from the culture filtrate was accomplished by adsorption on Hyflo Super-Cel, and elution with 0.075 M ammonium hydroxide.

Purification of the enzyme was achieved by precipitation at 100 per cent ammonium sulfate saturation, after preliminary removal of impurities at the 50 per cent saturation level.

Yields and activity of the enzyme are specified.

BIBLIOGRAPHY

1. Abraham, E. P., and Chain, E., *Nature*, 146, 837 (1940).
2. Harper, G. J., *Lancet*, 1, 569 (1943).
3. Smith, W., and Smith, M. M., *Lancet*, 1, 809 (1945).
4. Proom, H., *Brit. J. Exp. Path.*, 26, 98 (1945).
5. Duthie, E. S., *Brit. J. Exp. Path.*, 25, 96 (1944).
6. Benedict, R. G., Schmidt, W. H., and Coghill, R. D., *Arch. Biochem.*, 8, 377 (1945).
7. McQuarrie, E. B., and Liebmann, A. J., *Arch. Biochem.*, 5, 307 (1944).
8. Murtaugh, J. J., and Levy, G. B., *J. Am. Chem. Soc.*, 67, 1042 (1945).
9. Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 47, 199 (1944).
10. Woodruff, H. B., and Foster, J. W., *J. Bact.*, 49, 7 (1945).

THE IDENTIFICATION OF COMPOUND B, A SUBSTANCE OCCURRING IN OX BILE, AS ALLOPREG-NANEDIOL-3(β),20(β)

By W. H. PEARLMAN*

(From the Department of Experimental Medicine, Jefferson Medical College, Philadelphia)

The author has described (1) the isolation of Compound B, m.p. 192–193°, from the non-ketonic, digitonin-precipitable fraction of the neutral, non-saponifiable material obtained from ox bile. The empirical formula $C_{21}H_{36}O_2$ was assigned and the substance tentatively regarded as an (allo) pregnane derivative containing a 3(β)-hydroxyl group. Lack of material prevented further structural elucidation.

After an unsuccessful attempt to obtain additional amounts of Compound B from another batch of ox bile hydrolysate, recourse was had to direct comparison of the melting points of Compound B and its dibenzoate with certain pregnanediol isomers and their respective derivatives (Table I). It is obvious from Table I that Compound B is neither allopregnanediol-3(β),20(α) nor pregnanediol-3(β),20(β). Suspicion rested therefore on allopregnanediol-3(β),20(β) or possibly on the pregnanediol product, m.p. 189–190.5°, uncorrected, described by Butenandt and Müller (6). Since only the benzoyl derivative of Compound B was available, the preparation of the dibenzoates (not previously described) of the compounds under consideration was undertaken. Allopregnanediol-3(β),20(β) was prepared directly from Δ^5-pregnenol-3(β)-one-20 by hydrogenation in the presence of platinum oxide. Comparison of the melting points of the dibenzoates of the pregnanediol isomers in question with each other and with the dibenzoate of Compound B clearly favors allopregnanediol-3(β),20(β). Indeed, admixture of Compound B with the latter substance gave no depression in melting point, and the same was the case with the respective benzoyl derivatives.

Of incidental interest are the unexpected results obtained when progesterone was reduced in the presence of palladium catalyst (a reaction not previously described) in order to obtain pregnanedione-3,20, an intermediate required for the preparation of pregnanediol-3(β),20(α) by the method of Butenandt and Müller (6). Allopregnanedione-3,20 was obtained as the chief product; the mother liquors yielded a crystalline

* Present address, Department of Biochemistry, Jefferson Medical College, Philadelphia.

mixture, presumably of allopregnanedione and pregnanedione which could not be separated by chromatography. It is interesting that similar reduction of a compound closely related to progesterone, namely $\Delta^{4,16}$-pregnanedienone-3,20, was reported by Marker et al. (8) to yield pregnanedione and a small amount of progesterone. In the bile acid series, Schoenheimer and Berliner (9) observed that partial hydrogenation of 3-keto-Δ^4-cholenic acid with active palladium gave a mixture of 3-keto-cholanic acid and a small amount of 3-ketoallocholanic acid.

Allopregnanediol-3(β),20(β) probably arises by the *in vivo* reduction of allopregnanol-3(β)-one-20, a substance which has been isolated from the

TABLE I

Comparison of Compound B with Isomers of Pregnanediol

Steroid	M.p.*	M.p. of dibenzoate*
	°C.	°C.
Compound B	192–193 (1)	234–235 (1)
Allopregnanediol-3(β),20(β)	195–196 (2)	
	192–194 (3)	
	193 (4)	
	193–194	239–239.5
Pregnanediol-3(β),20(α)	182 (5, 1)	167–168 (1)
	189–190.5	
	(Uncorrected) (6)†	
	190–191†	169.5
Allopregnanediol-3(β),20(α)	218 (5)	
Pregnanediol-3(β),20(β)	176 (7)	

* The figures in parentheses are bibliographic reference numbers.
† Possible isomerism at C_{17} is not excluded.

corpus luteum and the adrenal glands. It is curious that the first pregnane-diol isomer to be isolated from bile contains a C_{20}-β-hydroxyl group, whereas the pregnanediol isomers and the pregnenediol which have been isolated from urinary sources (for a summary cf. (10)) possess a C_{20}-α-hydroxyl group. It was the uniform configuration of the C_{20} hydroxyl group in the latter compounds which led Marker et al. (5) to designate it arbitrarily as the α or natural configuration. Apparently the bile can serve as a medium for the excretion of C_{21} steroid hormone metabolites. In this connection, it is pertinent to mention that in a metabolism study by Pearlman and Pincus (11) pregnanediol-3(α),20(α) was isolated from the bile and urine of a postmenopausal woman after the oral administration of Δ^5-pregnenol-3(β)-one-20.

The above mentioned failure to isolate Compound B from ox bile may mean that this substance does not regularly occur in the bile, since identical

source material was not available to the author. The non-ketonic, digitonin-precipitable material obtained in the course of this investigation yielded complex crystalline mixtures from which a product, m.p. 175–176°, was obtained in amount insufficient for analysis; it gave a negative Rosenheim test (90 per cent aqueous trichloroacetic acid) at room temperature. The non-ketonic fraction, however, did yield a compound previously isolated from this source, namely Compound C.

<div align="center">EXPERIMENTAL</div>

Allopregnanediol-3(β),20(β) from Δ⁵-Pregnenol-3(β)-one-20—108 mg. (0.345 mM) of Δ^5-pregnenol-3(β)-one-20, m.p. 189–190°, were dissolved in 11 ml. of acetic acid and hydrogenated in the presence of 88 mg. of platinum oxide which had been previously reduced. 0.74 mM of hydrogen was taken up. The filtrate was evaporated *in vacuo;* traces of acetic acid were removed by repeated addition of alcohol and evaporation. The residue was repeatedly crystallized from alcohol, yielding 32 mg. of needles, m.p. 193.5–194°. The melting point was not raised by recrystallization from ethyl acetate. The diacetate melted at 141.5–142° (*cf.* m.p. 142–143° (2)).

Dibenzoate of Allopregnanediol-3(β),20(β)—25 mg. of the product, m.p. 193–194°, was dissolved in 1 ml. of pyridine and 0.2 ml. of benzoyl chloride was added. After standing at room temperature for 48 hours, the reaction mixture was poured into an excess of a dilute solution of sodium carbonate and heated for about 15 minutes on the steam bath. The granular product was washed with water, dried, and repeatedly crystallized from benzene-alcohol; 25 mg. of needles, m.p. 239–239.5°, were obtained.

<div align="center">$C_{35}H_{44}O_4$. Calculated, C 79.50, H 8.40; found, C 79.20, H 8.24</div>

Partial Reduction of Progesterone—481 mg. of progesterone, m.p. 128–129°, were dissolved in 15 ml. of absolute ethanol and hydrogenated in the presence of palladium on zirconium oxide (American Platinum Works). After 85 minutes the hydrogen uptake ceased, 1.15 mM equivalents of hydrogen having been absorbed. Crystallization from alcohol yielded 225 mg. of crystals, m.p. 190–198°; repeated crystallization from this solvent gave 142 mg., m.p. 198–201°. Chromatographic analysis of the mother liquors gave crystalline mixtures with melting points ranging from 111–170°.

Dibenzoate of Pregnanediol-3(β),20(α)—6 mg. of the crystalline product, m.p. 190–191°, which had been prepared by reducing pregnanol-3(β)-one-20 with isopropyl alcohol and sodium (6), were benzoylated in the usual way. The product, on repeated crystallization from alcohol, yielded 3 mg. of plates, m.p. 168–169.5°.

SUMMARY

Compound B, a substance which occurs occasionally in ox bile, has been identified as allopregnanediol-3(β),20(β).

BIBLIOGRAPHY

1. Pearlman, W. H., *J. Am. Chem. Soc.*, **66**, 806 (1944).
2. Marker, R. E., Kamm, O., Jones, D. M., and Oakwood, T. S., *J. Am. Chem. Soc.*, **59**, 614 (1937).
3. Marker, R. E., and Rohrmann, E., *J. Am. Chem. Soc.*, **62**, 898 (1940).
4. Butenandt, A., and Schmidt-Thomé, J., *Ber. chem. Ges.*, **72**, 1960 (1939).
5. Marker, R. E., Kamm, O., Wittle, E. L., Oakwood, T. S., Lawson, E. J., and Laucius, J. F., *J. Am. Chem. Soc.*, **59**, 2291 (1937).
6. Butenandt, A., and Müller, G., *Ber. chem. Ges.*, **71**, 191 (1938).
7. Marker, R. E., Kamm, O., and Jones, D. M., *J. Am. Chem. Soc.*, **59**, 1595 (1937).
8. Marker, R. E., Tsukamoto, T., and Turner, D. L., *J. Am. Chem. Soc.*, **62**, 2525 (1940).
9. Schoenheimer, R., and Berliner, F., *J. Biol. Chem.*, **115**, 19 (1936).
10. Pincus, G., and Pearlman, W. H., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **1** (1943).
11. Pearlman, W. H., and Pincus, G., *Federation Proc.*, **5**, 79 (1946).

EFFECT OF DEHYDRATION ON ENZYMIC DESTRUCTION OF CAROTENE IN ALFALFA*

BY H. L. MITCHELL AND H. H. KING

(*From the Department of Chemistry, Kansas State College, Manhattan*)

It has been demonstrated that lipoxidase is responsible for a considerable part of the destruction of carotene in alfalfa as it cures in the field (1). There have been no reports concerning the carotene-destroying ability of peroxidase, which is also present in alfalfa. This investigation was undertaken to determine whether these two oxidative enzymes are involved in the destruction of carotene that occurs during the storage of dehydrated alfalfa meal.

EXPERIMENTAL

Alfalfa was harvested when in early bloom. Part of the fresh material was blanched with steam at atmospheric pressure for 10 minutes. The blanched and unblanched materials were then dehydrated in a pilot plant dehydrator and were ground to pass through a 1 mm. screen. The samples were stored at −23° until used.

The temperature of the hot gases at the entrance of the dehydrator tunnel was about 550°. The temperature at the exit was 150°. These conditions are comparable to those which are used with many commercial dehydrators.

Lipoxidase and Peroxidase Activity—An extract of fresh alfalfa was prepared by dispersing 1 gm. of fresh tissue in 100 ml. of water with a Waring blendor. Extracts of the alfalfa meals were prepared by steeping 2 gm. of meal in 50 ml. of water for 1 hour and filtering. Into a 125 ml. Erlenmeyer flask were placed 2 ml. of the extract to be tested, 10 ml. of water, 10 ml. of pH 6.5 phosphate buffer, and 1 ml. of a solution consisting of approximatey 100 γ of carotene and 0.6 mg. of Wesson oil per ml. of acetone. The contents of the flask were mixed by swirling and the flask was placed in an incubator at 36°. After 1 hour 35 ml. of 95 per cent ethanol were added, followed by exactly 50 ml. of Skellysolve B. The flask was stoppered and shaken vigorously. The contents of the flask were poured into a separatory funnel, water was added, and the aqueous layer was removed. The Skellysolve B solution, containing the undestroyed carotene, was washed with water and was dried over anhydrous sodium sulfate. Carotene was

measured with a Beckman spectrophotometer at 4360 A. A blank determination was made by substituting an equal volume of water for the alfalfa extract. Lipoxidase activity was expressed as per cent destruction of the carotene that was added.

Peroxidase activity was observed qualitatively as described by Proctor (2). The test was performed on both heated and unheated extracts to guard against erroneous results due to oxidation of the benzidine reagent that may occur if iron or copper salts are present (2).

The results of these tests, presented in Table I, show that lipoxidase was inactivated by the dehydration process, while peroxidase was not. Peroxidase was inactivated by blanching, however.

Effect of Peroxidase on Carotene Destruction—Since the dehydrated meal gave a positive test for peroxidase, the experiment which follows was performed to determine whether alfalfa peroxidase possesses carotene-destroying activity.

TABLE I

Lipoxidase and Peroxidase Activity of Fresh, Dehydrated, and Blanched Dehydrated Alfalfa

Treatment	Lipoxidase, carotene destroyed	Peroxidase
	per cent	
Fresh..................................	62	Positive
Dehydrated...........................	0	"
Blanched dehydrated.................	0	Negative

10 gm. of the dehydrated alfalfa meal were steeped in 200 ml. of water for 1 hour. . The mixture was filtered and part of the resulting extract was heated for 10 minutes on a steam cone. Into a 125 ml. Erlenmeyer flask were placed 30 ml. of the extract to be tested, 15 ml. of water, and 5 ml. of a carotene solution consisting of approximately 100 γ of carotene per ml. of acetone. 4 drops of 3 per cent hydrogen peroxide were then added. After 1 hour the unaltered carotene was removed by adding 50 ml. of ethanol, transferring the mixture to a separatory funnel, and extracting four times with 40 ml. portions of Skellysolve B. The carotene was measured as previously described. Heated and unheated extracts were used both with and without the addition of hydrogen peroxide. This test differed from the qualitative peroxidase test in that carotene in acetone was added as the oxidizable material instead of benzidine in alcohol. The presence of the acetone did not inhibit the enzyme, since the benzidine test when 5 ml. of acetone were added was strongly positive.

The results of this experiment, presented in Table II, show that the

peroxidase which was extracted from dehydrated alfalfa meal did not cause the oxidation of carotene.

Effect of Blanching Prior to Dehydration on Retention of Carotene during Storage—Although peroxidase after extraction from the meal did not destroy carotene, it is possible that it can do so when in its natural relationship to other constituents in the meal. If this is the case, blanching before dehydration should result in better retention of carotene during storage of the meal.

TABLE II

Effect of Alfalfa Peroxidase on Carotene Destruction in Aqueous Solution

Treatment	Carotene recovered
	γ
Blank.	521
Heated extract.	523
Extract.	523
Heated extract $+ H_2O_2$.	527
Extract $+ H_2O_2$.	510

TABLE III

Effect of Blanching Prior to Dehydration on Carotene Retention during Storage of Alfalfa Meal

Treatment	Carotene after storage			Loss	
	0 mo.	1 mo.	2 mos.	1 mo.	2 mos.
	γ per gm.	γ per gm.	γ per gm.	per cent	per cent
Dehydrated.	228	175	126	23.2	44.7
Blanched dehydrated.	243	186	138	23.5	43.2

The meals were stored in the dark at room temperature (27–34°). Carotene was determined after 0, 1, and 2 months of storage by the method of Silker, Schrenk, and King (3).

It has been reported that peroxidase solutions which have been inactivated by heat regain their activity upon standing (4). Accordingly, the lipoxidase and peroxidase tests were repeated after 2 months of storage. No regeneration of either enzyme was detected.

The results of this experiment (Table III) show that blanching prior to dehydration did not result in greater retention of carotene. Since there was no regeneration of peroxidase activity during storage of the blanched dehydrated meal and since the dehydrated meal continued to show activity, these results are further evidence that alfalfa peroxidase is not responsible for carotene destruction in storage. Since lipoxidase also remained inac-

tive, these data indicate that carotene destruction in stored dehydrated alfalfa is probably not enzymic in nature.

SUMMARY

Alfalfa lipoxidase was inactivated by the dehydration process. Peroxidase was inactivated only when the alfalfa was blanched before dehydration.

Alfalfa peroxidase, either in aqueous extracts or in the meal, did not catalyze the oxidation of carotene.

There was no regeneration of either lipoxidase or peroxidase activity during storage of alfalfa meal over a 2 month period.

Blanching of alfalfa prior to dehydration did not increase the retention of carotene during storage. Carotene destruction during storage does not appear to be enzymic in nature.

BIBLIOGRAPHY

1. Mitchell, H. L., and Hauge, S. M., *J. Biol. Chem.*, **163,** 7 (1946).
2. Proctor, B. E., *Food Ind.*, **14,** 51 (1942).
3. Silker, R. E., Schrenk, W. G., and King, H. H., *Ind. and Eng. Chem., Anal. Ed.*, **16,** 513 (1944).
4. Bach, A., and Wilensky, B., *Biochem. Z.*, **226,** 482 (1930).

NEW FACTORS IN THE NUTRITION OF LACTOBACILLUS CASEI*

By M. L. SCOTT, L. C. NORRIS, AND G. F. HEUSER

WITH THE TECHNICAL ASSISTANCE OF LENA STRUGLIA

(From the Agricultural Experiment Station and the School of Nutrition,
Cornell University, Ithaca)

Studies of the nutritive requirements of the bacterium, Lactobacillus casei, have contributed much to our present knowledge of animal nutrition. It appears that the vitamin requirements of this microorganism are very similar to those of animals.

Landy and Dicken (1) reported a microbiological assay method for six B vitamins with Lactobacillus casei and a medium of essentially known composition. This work indicated that the vitamin requirements of L. casei were met by inclusion in the growth medium of riboflavin, biotin, pantothenic acid, pyridoxine, nicotinic acid, and folic acid. This method of assay was found to work successfully in many laboratories. However, in some laboratories good growth of L. casei could not be obtained on this medium.

Pollack and Lindner (2) reported a growth stimulant for Lactobacillus casei which was present in Wilson's peptone. They reported that glutamine was capable of replacing the peptone factor, but that glutamine itself could not be identical with the active factor in peptone since it is easily destroyed by mild alkaline hydrolysis while the factor in peptone is not.

Woolley (3) reported that certain hemolytic streptococci require an unknown factor for growth. Later, Sprince and Woolley (4) found that, under certain conditions, this factor is also required by a variety of bacteria, including Lactobacillus casei. These workers have tentatively named the factor strepogenin. Sprince and Woolley (5) have found strepogenin to be an integral part of certain purified proteins from which it can be freed most efficiently by incubation with trypsin. Their studies of the properties of this factor have led them to suggest that it may be a peptide. They have reported that strepogenin is dialyzable after trypsin digestion, that it is destroyed by acid hydrolysis, and that it is adsorbed to some extent by barium sulfate, but not by charcoal.

481

Attempts in this laboratory to determine folic acid with *Lactobacillus casei* assay with a basal medium containing all of the known growth essentials for *L. casei* failed when sulfuric acid-hydrolyzed, charcoal-treated Difco isoelectric casein was used as the source of the casein hydrolysate. Other sources of casein hydrolysate proved to furnish something which was lacking in the Difco casein and gave varying degrees of growth response when used in the medium for folic acid assay. These differing growth responses were most pronounced during the first 16 hours of incubation. The addition of a mixture of twenty amino acids to the Difco casein medium at a level of 2 mg. of each amino acid per assay tube did not produce any improvement in growth. This mixture contained all sixteen of the amino acids shown by Hutchings and Peterson (6) to meet the amino acid requirements of *L. casei* and, in addition, contained glycine, norleucine, proline, and hydroxyproline.

An investigation was undertaken, therefore, to discover a source of the missing growth factors which would be suitable for supplementing the basal medium for the determination of folic acid.

<div align="center">EXPERIMENTAL</div>

Since our basal medium was very similar to that used by Sprince and Woolley (4) for the determination of strepogenin, it was assumed that strepogenin was the factor not present in our basal medium.

With the hydrolyzed Difco casein basal medium plus adequate folic acid, a series of assays was made to determine the relative potency of various natural materials in terms of strepogenin. The composition of the basal medium is presented in Table I.

All samples were digested with trypsin prior to assay according to the method of Sprince and Woolley (5). A trypsin blank was run in each assay and the amount of strepogenin due to the trypsin present in each digest was subtracted to give the strepogenin content of the material itself.

Various levels of the material to be assayed were added to 5 ml. of the basal medium and then each assay tube was filled to a volume of 10 ml. with distilled water. The tubes were autoclaved for 10 minutes at 15 pounds pressure, cooled, and 1 drop of a sterile solution of pyridoxal containing approximately 200 millimicrograms per drop was added to each assay tube by means of a sterile micro burette. The pyridoxal was sterilized by filtration through a Seitz bacteriological filter.

The method of handling the *Lactobacillus casei* culture is of paramount importance in conducting a successful assay for strepogenin. It was found that, in order to obtain an optimum rate of growth of this microorganism during the first 16 hours on any medium, a culture of high metabolic activity is essential. In order to keep the culture in this active form, it is necessary

to use the following procedure. Each week, the culture of *Lactobacillus casei* is transferred from the agar stab into 10 ml. of a broth containing glucose, Bacto-tryptone, Difco yeast extract, fresh beef liver extract, and dipotassium phosphate. The composition and method of preparation of this broth are described by Nymon, Gunsalus, and Gortner (8). After 24 hours incubation in this broth, the organism is transferred to an agar stab

TABLE I

Basal Medium for Lactobacillus casei

Casein hydrolysate (norit A)*	10 gm.
l-Cystine (dissolved in HCl)	400 mg.
l-Tryptophane	400 "
Dextrose	40 gm.
Sodium acetate trihydrate	40 "
K_2HPO_4	5 "
Adenine, guanine, and uracil, each	20 mg.
Xanthine	20 "
Salt Solution B (Snell and Strong (7))	10 ml.
Adjust to pH 6.6–6.8, steam, filter through Celite	
Add	
Folic acid	4 γ
Pyridoxine	2400 "
Thiamine	400 "
Riboflavin	400 "
Niacin	1200 "
Ca pantothenate	800 "
Biotin	800 millimicrograms
p-Aminobenzoic acid	20 γ
Pyridoxal†	40 "
Make to	1000 ml.

* Sulfuric acid-hydrolyzed Difco isoelectric vitamin-free casein (100 gm. in 500 ml. of solution) is adjusted to pH 3.0. Add 10 gm. of norit A (norit A is activated immediately before use by autoclaving it for 3 hours at 20 pounds pressure); stir for 15 minutes at room temperature; filter and store in the refrigerator.

† Pyridoxal was sterilized by filtration and added aseptically after autoclaving assay tubes.

of the same composition but also containing 1.5 per cent agar and 0.1 per cent calcium carbonate. After a 24 hour incubation, these agar stabs are stored in the refrigerator for use throughout the following week.

The inoculum tube for the strepogenin assay is prepared by transferring by sterile needle from the agar tube to 10 ml. of the Difco casein basal medium to which have been added 50 mg. of charcoal-treated Difco Bacto-peptone and 20 mg. of potassium permanganate-treated whey extract. These supplements are prepared in the following manner.

10 gm. of Difco Bacto-peptone are dissolved in 100 ml. of distilled water. 10 gm. of Darco G-60 are added and the mixture is stirred for $\frac{1}{2}$ hour and filtered. This adsorption is repeated for two more $\frac{1}{2}$ hour periods and the final filtrate is used in making up 1 liter of medium. The whey extract is prepared by steaming a solution of 50 gm. of dried whey in 500 ml. of

TABLE II

Relative Strepogenin Content of Various Materials

Material	Potency*
	mg. units per gm.
Crude casein	4400
Dried buttermilk	2400
Purified casein	2000
Tryptone (Difco)	2000
Dried skim milk	1700
" whole "	1300
Blood fibrin (commercial)	1250
Dried brewers' yeast	1000
Soy bean meal	1000
Dried whey	950
Lactalbumin	760
Dried cereal grasses	550
Fish-meal	450
Meat scrap	450
Alfalfa meal	350
Liver meal	290
Dried egg albumin	240
Liver fraction L (Wilson)	210
Autoclaved dried egg albumin†	100
Gelatin	55
Corn-starch	35

* Potencies have been compared to that of dried brewers' yeast which has been assigned a value of 1000 mg. units per gm.

† Dried egg albumin autoclaved for 3 hours at 120°.

distilled water for 5 minutes and filtering. When the temperature of the extract reaches 50°, 5 gm. of $KMnO_4$ are added. After being stirred for 5 minutes, the brown precipitate is removed by filtration; 40 ml. of this solution are added per liter of medium.

After 24 hours, the inoculum is centrifuged, washed twice with 10 ml. portions of sterile physiological saline, and then resuspended in 10 ml. of the saline. The inoculum is diluted by pouring this 10 ml. suspension of cells into 500 ml. of sterile saline. Each assay tube is inoculated with 1 drop of this diluted cell suspension by means of a sterile micro burette. After 16 hours incubation, the strepogenin content is determined turbidi-

metrically with a Coleman model 11 spectrophotometer. The relative strepogenin content of a variety of materials is presented in Table II. The strepogenin content is expressed in mg. units per gm. based upon a comparison to the strepogenin content of strain S dried brewers' yeast which was assigned a value of 1000 mg. units per gm.

As reported by Sprince and Woolley (5), casein was found to be an excellent source of the factor. Dried brewers' yeast, dried whole milk, dried skim milk, dried buttermilk, dried whey, and soy bean meal were all found to be good sources of the factor. Difco tryptone was also found to be a very good source. On the other hand, egg albumin, gelatin, liver meal, fish-meal, meat scrap, Wilson's liver fraction L, fish press water, and alfalfa meal were all found to be relatively poor sources of the factor.

Since the factor required by *Lactobacillus casei* was found to be present in high amounts in materials such as casein, yeast, and soy bean meal, known to be good sources of the chick growth factor S of Schumacher, Heuser, and Norris (9), it was considered possible that factor S might be identical with the microbiological growth factor. Hill (10) has shown that factor S is not removed from solution by treatment with charcoal, a property also reported by Sprince and Woolley (5) for strepogenin.

In view of this, a solution of tryptone was treated three times for ½ hour each with an equal weight of Darco G-60 at pH 3.0 and the filtrate was assayed by *Lactobacillus casei*. In this case, although some growth resulted in the 16 hour incubation period, it plateaued at a submaximum level. Such a plateau strongly indicated that at least two required factors were present in the original tryptone solution, one of which was adsorbed, the other not adsorbed.

Chu and Williams (11), in studies on the "peptone factor" for *Lactobacillus casei*, have stated that the effect of peptone upon the growth of *L. casei* may be simulated by a mixture containing glutamine, *p*-aminobenzoic acid, and pyridoxal. Since our medium already contained *p*-aminobenzoic acid and pyridoxal, it was necessary to study only the effect of glutamine. Since, as Pollack and Lindner (2) have pointed out, glutamine is more readily hydrolyzed by autoclaving than is the peptone factor, it was thought that perhaps glutamine was necessary merely in the formation of some other growth essential, perhaps glutathione. Consequently, a study was conducted to compare the effects of glutamine and glutathione upon the growth of *L. casei*. It was found that, although glutamine and glutathione had very little effect upon growth when added alone, the growth of *L. casei* upon the tryptone filtrate plus glutathione was somewhat greater than that upon the tryptone filtrate alone. Even though glutathione caused an increase in growth of *L. casei* above that obtained with the tryptone filtrate alone, the response of the organism to these two factors

was still submaximum.   Thus it appeared that at least one more factor is required for normal growth of *L. casei*.

Studies conducted in this laboratory on chicks and hens have shown the need for growth and reproduction in the chicken of an unknown factor present in such animal products as liver, fish-meal, and milk products. It was considered possible that *Lactobacillus casei* might also need this unknown factor.   Since this factor is known to be soluble in 95 per cent alcohol, while strepogenin, prior to trypsin digestion, is not, an alcohol extract was made of dried whey.

TABLE III

*Effect of Darco-Treated Tryptone Filtrate, Potassium Permanganate-Treated Alcohol Extract of Whey, and Glutathione upon 16 Hour Growth Response of Lactobacillus casei**

Supplement	Level	Galvanometer readings†	
		Without glutathione	100 γ glutathione per tube
	mg.		
None		100	98
Tryptone filtrate	5	93	92
	10	94	92
	20	95	92
KMnO$_4$-treated alcohol extract	5	98	91
of whey	10	95	81
	20	92	80
KMnO$_4$-treated alcohol extract	20		
of whey			
+ tryptone filtrate	5	48.5	42
	10	39.0	34
	20	28.0	19.5

* Representative experiment conducted in quadruplicate.

† A reading of 100 represents no growth; a reading of approximately 20 represents maximum growth.

This is done by steaming 50 gm. of dried whey with 100 ml. of 95 per cent ethanol for $\frac{1}{2}$ hour and filtering.   This extraction is repeated for two more $\frac{1}{2}$ hour periods and the combined filtrates are taken almost to dryness on a steam bath and then made to 500 ml. with distilled water and filtered.

In the first assays this preparation, when added alone, was found to produce fair growth of *Lactobacillus casei*.   However, later it was found that the "animal protein factor" was stable to oxidation with a small amount of potassium permanganate, while strepogenin was readily destroyed by this procedure.   Accordingly, traces of strepogenin present in the alcohol extract of whey were removed by treatment with KMnO$_4$.

This permanganate treatment is carried out in the following manner.

The aqueous solution obtained after alcohol extraction of the whey is heated to 50° and treated with 5 gm. of $KMnO_4$ for 5 minutes. The pH of the solution is adjusted to 7.0 and a solution of 30 per cent hydrogen peroxide is added, drop by drop, until the violet color of the permanganate has disappeared. The resulting brown precipitate is filtered off and the filtrate is ready for use. Very little or no growth was obtained when this permanganate-treated alcohol extract of whey or the tryptone filtrate was supplied singly. Good growth was obtained with a combination of these two preparations. However, a maximum rate of growth of *Lactobacillus casei*, over a 16 hour growth period, was obtained only when glutathione was supplied in addition to these two preparations. These results are presented in Table III

DISCUSSION

Evidence has been presented showing that under certain conditions *Lactobacillus casei* requires, in addition to the known growth essentials, strepogenin, a factor present in animal products, and glutathione in order to maintain an optimum rate of growth during the first 16 hours of incubation.

The fact that hydrolysates of samples of casein from different sources vary widely in their content of strepogenin probably accounts for the failure of many workers to recognize the need of strepogenin for the growth of *Lactobacillus casei*.

Since the properties and distribution in natural materials of strepogenin and the chick growth factor S of Schumacher, Heuser, and Norris (9) are similar, it is possible that these two factors are identical. Evidence to this effect, based upon chick growth studies, is being presented in another report.

The growth-promoting effect of the factor present in the potassium permanganate-treated alcohol extract of whey could be shown only when it was included in the medium together with a source of strepogenin freed of this factor by charcoal adsorption. Since this factor appears to be present in most casein hydrolysates, it was found necessary to treat the casein hydrolysate with charcoal in order to reveal the growth-promoting effect of the factor. Whey, fresh beef liver, Wilson's solubilized liver, and condensed fish press water have all been found to be good sources of this factor. It can be extracted from all of these materials by 95 per cent ethanol. It is readily adsorbed at pH 3.0 on either Darco G-60 or norit A and can be eluted by a 10 per cent solution of ammonium hydroxide in 95 per cent ethanol. Strepogenin is not adsorbed under these conditions. The factor present in animal products is stable to heat and oxidation. Strepogenin is destroyed by prolonged autoclaving and by mild oxidation.

The properties and the distribution of the whey factor indicate that it may be identical with the factor of animal origin required for normal reproduction in hens. Evidence for this hatchability factor was probably first presented by Byerly, Titus, and Ellis (12) in 1933. Since that time a great deal of work has been done showing the need of such a factor for chick growth and for reproduction in hens on diets containing protein from vegetable sources only. This factor has been referred to from time to time as the "animal protein factor."

The effect exerted by glutathione upon the growth of *Lactobacillus casei* is usually not as pronounced as that produced by the other two factors. However, it always produces a definite improvement in growth and, in all assays for the other factors, glutathione has been included in the basal medium. The varying responses to the addition of glutathione may be due to the presence of varying amounts of this substance in the basal medium, or to a varying rate of synthesis of this substance by *Lactobacillus casei*.

It has been found that by including the Darco filtrate of tryptone, the potassium permanganate-treated whey extract, and glutathione in the basal medium and omitting folic acid a 16 hour assay may be conducted for this vitamin by using the procedure outlined for the strepogenin assay. By suitable modifications of the procedures for preparation of the medium and the supplements containing the unknown factors, it appears highly probable that satisfactory assay procedures could be developed for the other members of the vitamin B complex required by *Lactobacillus casei*.

SUMMARY

Evidence has been presented showing that, under certain conditions, *Lactobacillus casei* requires, in addition to the known growth essentials, (1) strepogenin, (2) a factor associated chiefly with animal products, and (3) glutathione in order to maintain an optimum rate of growth during the first 16 hours of incubation.

The assay procedures used in the determination of strepogenin and the factor of animal origin have been described.

The application of the findings presented in this report to *Lactobacillus casei* assays for other members of the vitamin B complex has been discussed.

BIBLIOGRAPHY

1. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
2. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **147**, 183 (1943).
3. Woolley, D. W., *J. Exp. Med.*, **73**, 487 (1941).
4. Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).
5. Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, **67**, 1734(1945).
6. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
7. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
8. Nymon, M. C., Gunsalus, I. C., and Gortner, W. A., *Science*, **102**, 125 (1945).
9. Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, **135**, 313 (1940).
10. Hill, F. W., Thesis, Cornell University (1944).
11. Chu, E. J. H., and Williams, R. J., *J. Biol. Chem.*, **155**, 9 (1944).
12. Byerly, T. C., Titus, H. W., and Ellis, N. R., *J. Nutr.*, **6**, 225 (1933).

HYDROLYSIS OF RIBONUCLEIC ACID WITH PHOSPHO-ESTERASE FROM CALF INTESTINAL MUCOSA

By CHARLES A. ZITTLE

(From The Biochemical Research Foundation, Newark, Delaware)

The hydrolysis of ribonucleic acid by an enzyme preparation from calf intestinal mucosa has been followed manometrically (1) by the liberation of CO_2 from a $NaHCO_3$ medium. The liberation of secondary phosphoric acid groups by a phosphodiesterase presumably was measured. This enzyme preparation was also a potent phosphomonoesterase (alkaline phosphatase).

The above phosphodiesterase was active with very low substrate concentrations (1) in contrast with the phosphodiesterase ribonucleinase (1, 2). This property permitted the enzyme to be used quantitatively to measure the secondary phosphoric acid groups liberated on the hydrolysis of ribonucleic acid, which has been done both manometrically and titrimetrically. In addition, the course of the enzymatic hydrolysis of the nucleic acid was determined from the increases in phosphorus soluble in the uranium reagent and inorganic phosphate.

EXPERIMENTAL

Preparation of Enzyme—Calf intestinal mucosa was obtained, treated with trypsin, and filtered as described for the preparation of alkaline phosphatase (3). The filtrate was precipitated with 600 gm. of ammonium sulfate per liter; the precipitate was collected on a Büchner funnel and dried in a vacuum desiccator. This product, about 70 per cent of which was ammonium sulfate, contained 1.4 units (see below) of phosphodiesterase per 10 mg. and 50.8 units of phosphomonoesterase per 10 mg., assayed by the method of Huggins and Talalay (4) with magnesium added. Approximately 5-fold increase in the activity of both was readily accomplished by the following procedure. 2.0 gm. are dissolved in 80 cc. of water with the addition of 1.0 cc. of N NaOH. The solution is centrifuged and dialyzed in a Visking tube against three 2 liter portions of water at 7° over a period of 24 hours. The precipitate which forms is discarded and the solution is treated with 2 volumes of cold acetone and 3.0 cc. of 2 N sodium acetate. The precipitate which forms on addition of the latter is dissolved in 50 cc. of water, treated four successive times with 5.0 cc. portions of $C\gamma$ alumina (5) (14.6 per cent solids, wet weight), and finally precipitated with 2 volumes of acetone and 1.0 cc. of 2 N sodium

acetate.   The precipitate is washed once with acetone and dried in a stream of air.   The resulting white powder, about 17 per cent of the starting material, contains almost all of the original activity, is completely soluble in water, and assays 8.0 units of phosphodiesterase and 218 units of phosphomonoesterase per 10 mg.   This material was used in the studies which follow.

Determination of Phosphodiesterase Activity—The activity was determined with Warburg equipment at 37°.   The enzyme was placed in the side arm of Warburg flasks with the following reagents in the bottom: 0.8 cc. of 0.5 M $NaHCO_3$, 1.0 cc. of 2 per cent ribonucleic acid (sodium nucleinate or the free acid after purification (6)) adjusted to pH 8.0 and
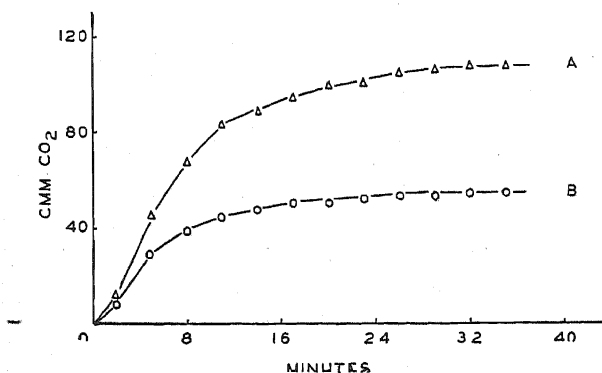
FIG. 1. Evolution of CO_2 by the action of phosphodiesterase on various concentrations of ribonucleic acid.   4.0 mg. of enzyme were used in each experiment under the conditions employed for the determination of the activity.   Ribonucleic acid Sample (EA$_{II}$-2), mg.; Curve A, 4.0, Curve B, 2.0.

water to make the total volume 3.5 cc.   An atmosphere of 5 per cent CO_2-95 per cent N_2 was employed; the pH was 8.14.   A liberation of CO_2 proportional to the amount of the enzyme was obtained when the rate was within 100 c.mm. per 10 minutes.   A unit of enzyme was defined as the amount liberating 50 c.mm. of CO_2 in 10 minutes under the above conditions.

Quantitative Use of Phosphodiesterase—The amount of nucleic acid used in the above assay (20.0 mg.) is in excess and gives a linear evolution of CO_2 for more than 30 minutes.   With smaller amounts of nucleic acid the release of CO_2 initially is large and decreases and finally stops (Fig. 1). Phosphate determinations have shown that the substrate is completely dephosphorylated.   The level of CO_2 production reached is a measure of the acid groups released but its exact estimation is subject to several corrections.   Corrections were made for the retention of CO_2 due to carbo-

nate ion and buffers ((7) pp. 125, 207) (determined by introducing a known amount of citric acid into the same system with the enzyme inactivated by heating 5 minutes at 100°) and for the dilution effect on adding the enzyme ((7) p. 216). In experiments performed under the conditions employed for the determination of activity both of these corrections were made. Experiments were also performed with 0.4 cc. of 0.5 M NaHCO$_3$ and an atmosphere of 2 per cent CO$_2$-98 per cent N$_2$ (pH 8.29). Under these conditions the dilution effect was negligible but the effect due to carbonate ion was quite large. Some experiments were performed with

TABLE I

Liberation of Secondary Phosphoric Acid Groups from Various Samples of Ribonucleic Acid, in Terms of Evolution of CO$_2$, by Action of Phosphodiesterase

Sample* No.	Nucleic acid P insoluble in uranium reagent (MacFadyen (9))		CO$_2$ evolved from 2.5 mg. nucleic acid		Ratio, CO$_2$ found to CO$_2$ theoretical
			Theoretical†	Found‡	
	mg. per 2.5 mg.	*moles per 2.5 mg. × 10⁶*			
EA$_{II}$-2	0.166	5.36	120	101.9	0.85
E$_I$, purified	0.185	5.98	134	95.2	0.71
M$_I$	0.183	5.91	132	103.5	0.78
EA$_{III}$, purified	0.204	6.48	146	109.0	0.75

* These samples were obtained commercially: EA, Eimer and Amend; E, Eastman; M, Merck.

† Theoretical for one secondary phosphoric acid group for each atom of phosphorus.

‡ Average of values obtained with 2.0 to 5.0 mg. of nucleic acid.

0.2 cc. of 0.5 M NaHCO$_3$ and an atmosphere of 5 per cent CO$_2$-95 per cent N$_2$ (pH 7.62). For these experiments the above corrections were negligible but at this pH adenosine deaminase which was present in the enzyme preparation (8) was active and in consequence the results were low. The results were corrected by the deamination obtained with an equivalent amount of mononucleotides prepared by the alkaline hydrolysis of nucleic acid.[1] In some experiments at this pH the deaminase was inhibited with silver (8) with like results.

The results obtained under the different conditions with several samples of nucleic acid are summarized in Table I. The data obtained by each method were reproducible within ±4 per cent, the data by the different methods agreed within ±7.5 per cent. The ratios in the last column are equivalent to the number of acid groups liberated for each atom of nucleic acid phosphorus; for consideration of the number per tetranucleotide

[1] Zittle, C. A., *J. Franklin Inst.*, **242**, 221 (1946).
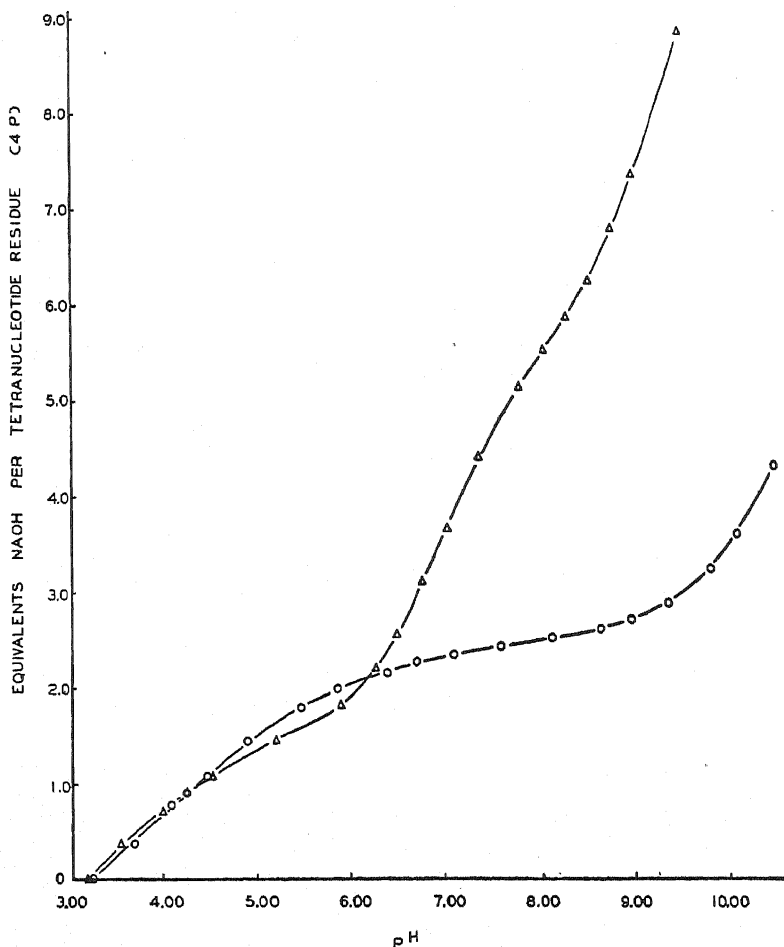
FIG. 2. Titration curves of ribonucleic acid before and after hydrolysis with phosphoesterase from calf intestinal mucosa. 0.50 gm. of nucleic acid (Sample EA$_{II}$-2) was hydrolyzed with 50.0 mg. of enzyme in a volume of 50.0 cc. for 24 hours at 25° with chloroform present. Occasionally N NaOH was added to bring the pH above 8.0. The completion of hydrolysis was confirmed by the liberation of phosphoric acid. Titration of the unhydrolyzed sample was performed with a similar solution with the enzyme inactivated (5 minutes at 100°). Both solutions were titrated to about pH 3.0 with N HCl and the back titration performed with 0.1 N NaOH. The equivalents of nucleic acid were based on the P insoluble in the uranium reagent (1 mole = 4 P). Curve A, unhydrolyzed; Curve B, hydrolyzed nucleic acid.

residue this ratio should be multiplied by 4. In comparing the results for the different samples of nucleic acid it should be kept in mind that they differ in degree of polymerization; for example, only 3.8 per cent of Sample

EA$_{II}$-2 will dialyze through a cellophane membrane, whereas 15.8 per cent of Sample M$_I$ and 18.3 per cent of Sample E$_I$, purified, will dialyze.[1]

Titration of Ribonucleic Acid before and after Enzymatic Hydrolysis—The unhydrolyzed and hydrolyzed nucleic acids were titrated over the pH range 3.0 to 10.5 to measure the release of secondary phosphoric acid
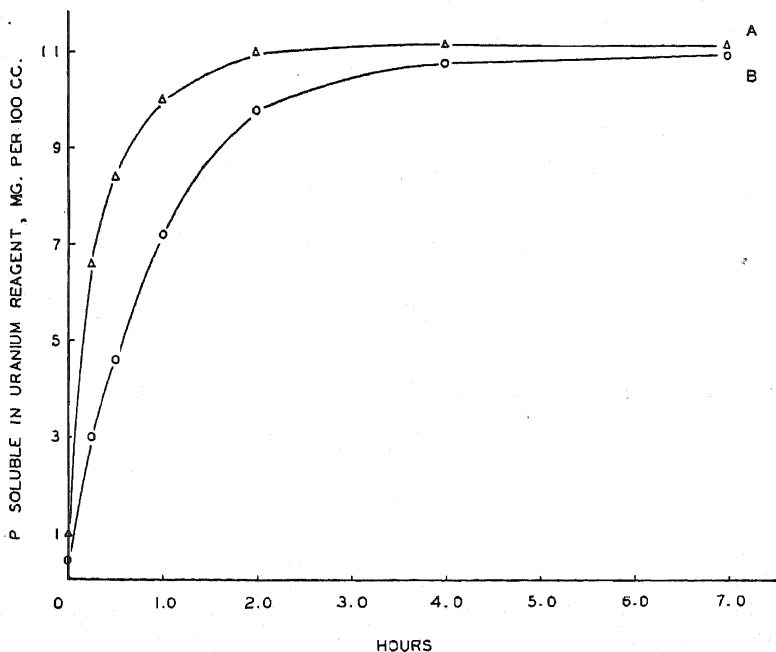
FIG. 3. Enzymatic hydrolysis of ribonucleic acid measured by solubility in uranium reagent and liberation of inorganic phosphate. 75.0 mg. of nucleic acid (Sample EA$_{II}$-2) were dissolved in 30.0 cc. of water and 12.0 cc. of 0.1 M NaHCO$_3$. 24.0 mg. of the enzyme in 5.0 cc. of water were added and a sample (total P = 0.11 mg. per cc.) taken immediately and mixed with an equal volume of uranium reagent. The precipitate was removed by centrifuging and total P (Curve A) and inorganic phosphate P (Curve B) determined on the supernatant fluid by the method of King (13).

groups. This was also estimated from the NaOH required to restore the pH of the hydrolysate to its initial value of ,8.70. The titration curves obtained (a duplicate experiment gave identical results) with the nucleic acid Sample EA$_{II}$-2 are shown in Fig. 2. The point of inflection for the unhydrolyzed nucleic acid was at 2.45 equivalents and pH 7.5 and for the hydrolysate 5.9 equivalents and pH 8.2. The net increase is 3.45 equivalents per tetranucleotide residue. The equivalents of base required to restore the pH of the hydrolysates to the initial pH were 3.76 and 3.85

for two experiments. The slight dip in Curve B in the neighborhood of pH 5.5 may be in consequence of the deamination of adenosine (pK′ = 3.45 (10) p. 213). The hydroxyl group which is formed by deamination has a pK′ of 8.75 (11) and, at pH 8.2, 22 per cent of it would be titrated; however, this much of the 0.25 equivalent of tetranucleotide involved is inappreciable.

Course of Enzymatic Hydrolysis of Ribonucleic Acid: Solubility in Uranium Reagent and Inorganic Phosphate—The action of the enzyme preparations on ribonucleic acid was also followed by determining the solubility of the nucleic acid in the uranium reagent (9, 12), in which tetranucleotides and larger components of nucleic acid are insoluble, and by determining the release of inorganic phosphate. The course of hydrolysis under conditions that gave complete hydrolysis in less than 7 hours at 20–25° is shown in Fig. 3. The early lag of the inorganic P at about one-half of the value of the soluble P (mononucleotides) probably represents the difference in the rates of hydrolysis of purine and pyrimidine nucleotides.

DISCUSSION

The number of secondary phosphoric acid groups liberated from ribonucleic acid by complete enzymatic hydrolysis is in fair agreement by manometric measurement and by titration. The number exceeds three per tetranucleotide residue (four P) and approaches four as a maximum with the most highly polymerized nucleic acid preparations.

Fletcher, Gulland, and Jordan (14) have postulated a formula for a tetranucleotide which contains a triester of phosphoric acid. The manometric procedure would not differentiate between a secondary and a primary phosphoric acid group but the titration experiment which measures only secondary phosphoric acid seems definitely to exclude such a structure.

The presence of more than three secondary phosphoric acid bonds per tetranucleotide residue is most in accord with the formula proposed by Levene ((10) p. 274); however, a simple straight chain structure repeating the tetranucleotide unit would not account for the specificity of ribonucleinase which hydrolyzes only about one-third of ribonucleic acid (15, 12) and yet releases all four of the mononucleotides (16).

The complete liberation of phosphoric acid from ribonucleic acid by the mucosa enzyme is consistent with the release of more than three secondary phosphoric acid groups per tetranucleotide residue. Gulland and Jackson (17) obtained only a 75 per cent dephosphorylation of ribonucleic acid by the action of an enzyme preparation with di- and monoesterase activity. In a later paper (18) they report that Boehringer and Merck nucleic acids were 75 per cent dephosphorylated by their enzymes but that the

British Drug Houses nucleic acid was 100 per cent dephosphorylated under the same conditions. This difference was being investigated further. Others (19, 3) have obtained complete dephosphorylation of ribonucleic acid with enzyme preparations from calf intestinal mucosa.

SUMMARY

Ribonucleic acid was completely hydrolyzed, with the liberation of all of the phosphoric acid, by an enzyme preparation from calf intestinal mucosa. The number of acid groups liberated during the hydrolysis was estimated by manometric and titrimetric procedures. It was found that the number of secondary phosphoric acid groups liberated from the most highly polymerized preparations of nucleic acid approached four per tetranucleotide residue.

BIBLIOGRAPHY

1. Zittle, C. A., *J. Franklin Inst.*, **241**, 379 (1946).
2. Bain, J. A., and Rusch, H. P., *J. Biol. Chem.*, **153**, 659 (1944).
3. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).
4. Huggins, C., and Talalay, P., *J. Biol. Chem.*, **159**, 399 (1945).
5. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, 10th edition, 253 (1931).
6. Zittle, C. A., *J. Biol. Chem.*, **160**, 527 (1945).
7. Warburg, O., The metabolism of tumours, translated by Dickens, F., New York (1931).
8. Zittle, C. A., *J. Biol. Chem.*, **166**, 499 (1946).
9. MacFadyen, D. A., *J. Biol. Chem.*, **107**, 297 (1934).
10. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York (1931).
11. Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, **70**, 243 (1926).
12. Zittle, C. A., *J. Biol. Chem.*, **163**, 119 (1946).
13. King, E. J., *Biochem. J.*, **26**, 292 (1932).
14. Fletcher, W. E., Gulland, J. M., and Jordan, D. O., *J. Chem. Soc.*, 33 (1944).
15. Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).
16. Loring, H. S., and Carpenter, F. H., *J. Biol. Chem.*, **150**, 381 (1943).
17. Gulland, J. M., and Jackson, E. M., *J. Chem. Soc.*, 1492 (1938).
18. Falconer, R., Gulland, J. M., Hobday, G. I., and Jackson, E. M., *J. Chem. Soc.*, 907 (1939).
19. Cohen, S. S., and Stanley, W. M., *J. Biol. Chem.*, **142**, 863 (1942).

ADENOSINE DEAMINASE FROM CALF INTESTINAL MUCOSA

By CHARLES A. ZITTLE

(From The Biochemical Research Foundation, Newark, Delaware)

In the course of studying the phosphoric acid esterases from calf intestinal mucosa (1, 2) the preparations were found to contain a potent deaminase for adenosine. The method of preparation is simple and the procedure indicates that this enzyme is quite stable. This deaminase is probably identical with that recently prepared by Brady (3) from the same material by a somewhat different procedure. In the present studies the action of the enzyme was followed manometrically; the utilization of this procedure for the estimation of adenosine is suggested.

EXPERIMENTAL

Preparation of Enzyme—The enzyme preparation described elsewhere (2) was used throughout these studies. The essential steps in the preparation are treatment of the mucosa with proteolytic enzymes, precipitation with 2 volumes of acetone with the addition of sodium acetate, treatment with $C\gamma$ alumina which removes impurities, and finally precipitation and drying with acetone.

Specificity of Deaminase—Ribonucleic acid, desoxyribonucleic acid (depolymerized (1)), and adenylic acid are deaminated at pH 7.6 but not at pH 5.9, whereas adenosine is deaminated at both pH values. Since the enzyme preparation contains phosphoesterases (2) which are inactive at the lower pH it was concluded that the first compounds are acted on only after dephosphorylation and that adenosine and desoxyriboadenosine were the specific substrates. The enzyme does not act on adenine.

Determination of Activity and Quantitative Application of Deaminase—The activity was determined by direct estimation of the NH_3 liberated and also manometrically from the absorption of CO_2 into the system made alkaline by the liberation of NH_3.

The direct procedure was used to demonstrate that NH_3 was formed from adenosine by the action of the enzyme and to determine the extent of the reaction. The enzyme was allowed to act on the substrate, the solution was then made alkaline with K_2CO_3, and the NH_3 was steam-distilled from a micro-Kjeldahl apparatus into the standard acid. Negligible blanks were obtained with the concentration of alkali provided by the K_2CO_3 with adenosine. In a typical experiment[1] 90.7 per cent of the amino N was found as free NH_3.

---

[1] The amount of adenosine used was based on the N content of the sample.
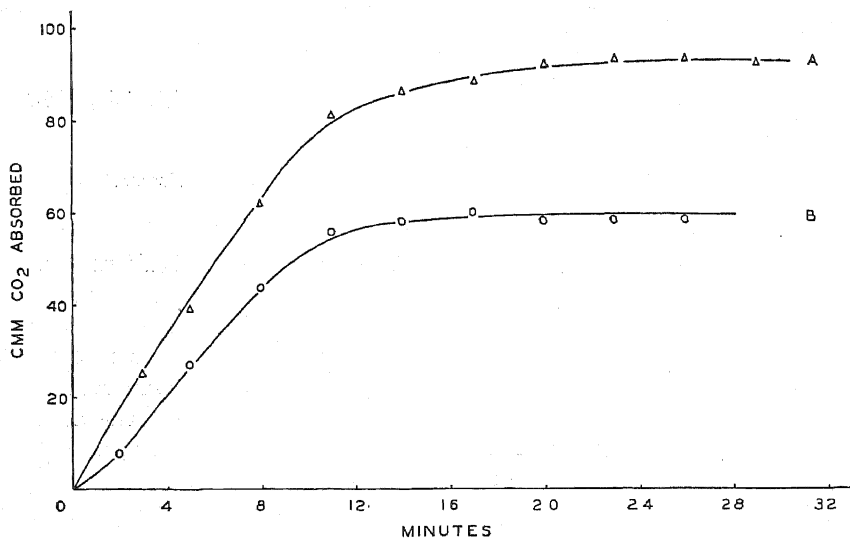
FIG. 1. Quantitative liberation of NH₃ from adenosine. The experiments were performed at 37° with an atmosphere of 5 per cent CO_2-95 per cent N_2. The Warburg flasks contained 0.2 cc. of 0.5 M $NaHCO_3$, adenosine, and 0.16 mg. of enzyme (in the side arm); the total volume after mixing was 3.5 cc. The pH was 7.62. Adenosine, Mg.; Curve A, 1.39, Curve B, 0.93.
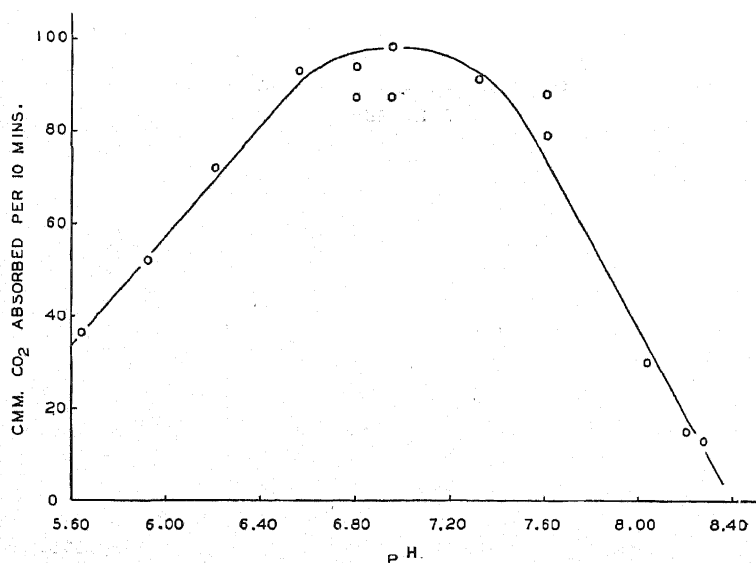
FIG. 2. Deaminase activity in relation to pH. The experiments were performed at 37° with 5.0 mg. of adenosine and with 0.16 mg. of enzyme (in the side arm); the total volume was 3.5 cc. $NaHCO_3$ and CO_2 were varied to give the pH values indicated; the points below pH 7.20 were obtained with an atmosphere of 100 per cent CO_2; those above were obtained with 5 per cent CO_2-95 per cent N_2.

500

The Warburg equipment was used for the manometric procedure. The absorption of CO_2 with excess substrate (5.0 mg. of adenosine per 3.5 cc.) was proportional to the amount of enzyme used (0.04 to 0.16 mg.). The deaminase is active with small amounts of substrate and so it can be used in quantitative experiments with adenosine. Experiments of this type are illustrated in Fig. 1. In this experiment 79 and 76 per cent, respectively, of the adenosine was accounted for by the c.mm. of gas absorbed.
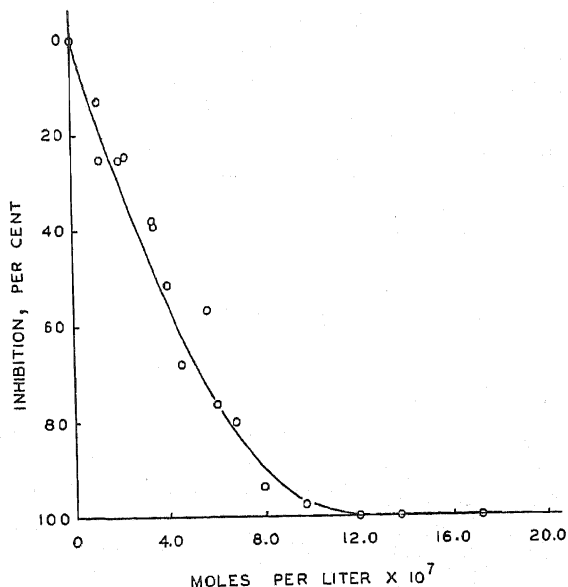
FIG. 3. Effect of silver on deaminase. In these experiments the enzyme (0.16 mg.) and the silver were in the bottom of the flask and the adenosine (5.0 mg.) was in the side arm. A stock solution of 0.002 M silver nitrate was diluted 1:500 or 1:1000 just before use and amounts added to give the indicated moles per liter. The total volume in the flask was 3.5 cc.

At pH 7.62, 2 per cent of NH_4OH exists as NH_3 (pK = 9.26) which would not absorb CO_2. A correction must be made also for the acidic hydroxyl group resulting from the deamination (pK of inosine = 8.75 (4)); at pH 7.62, 0.07 equivalent of CO_2 would be liberated by this group for each equivalent of adenosine deaminated. These corrections would increase the above figures to 88 and 85 per cent, values which are in fair agreement with the data obtained by direct measurement of the NH_3 formed.

Optimum pH of Deaminase—The optimum pH for the activity of the deaminase was determined as described under Fig. 2. There is a broad optimum at pH 7.0.

Effect of Silver on Deaminase—The effect of silver on the deaminase was determined as described under Fig. 3, where the results are shown.

<div style="text-align:center">DISCUSSION</div>

The deaminase is rather striking in its resistance to proteolytic enzymes and its stability when precipitated and dried with acetone. Schmidt (5) had observed that treatment with proteolytic enzymes[2] was useful in isolating adenosine deaminase from muscle and had noted that Cγ alumina would not absorb the deaminase. The enzyme described by Brady (3) from calf intestinal mucosa deaminated both adenosine and desoxyribo-adenosine; the essential steps in its purification were the extraction of acetone-dried mucosa with water, precipitation of inactive proteins with salicylic acid, and precipitation and drying with acetone. 1 gm. of this material liberated 180 mg. of NH_3-N per minute at 18°, or, with a factor of 2 for each 10° rise in temperature, 684 mg. per minute at 37°. The preparation used in the present studies caused the absorption of 61,200 c.mm. of CO_2 per minute per gm., equivalent to 37.8 mg. of NH_3-N per minute. Further purification was attempted with the use of salicylic acid[3] without success. The use of acid does not seem to be a promising purification procedure, since Brady (3) has found that exposure of the enzyme to pH 3.0 for 30 minutes will completely inactivate it.

The pH optimum of 7.0 obtained with the present preparation by the manometric method at 37° is somewhat higher than 6.2 for the enzyme from rabbit liver (6) and 6.5 for the enzyme in laked rabbit blood (7).

The effect of silver on the deaminase is of interest because of the small amount required and it is useful because by the addition of silver the deaminase can be inhibited when it is desired to use the phosphoesterases in these preparations for preparing adenosine from nucleic acid (8, 9). The deaminase in its sensitivity to silver is comparable to urease. Sumner and Myrbäck (10) found that crystalline urease (2.5 mg. per liter) was 50 per cent inhibited by less than 0.3×10^{-7} moles of silver per liter. The deaminase (45 mg. per liter) was 50 per cent inhibited by 4.0×10^{-7} moles of silver per liter. In comparing the results it should be kept in mind that the deaminase is far from pure.

Adenosine can readily be determined manometrically with the deaminase.

---

[2] Schmidt (5) chose to use preparations of papain rather than trypsin for this treatment because the latter contained adenosine deaminase. Manometric assays of Difco trypsin, 1:250 (1.0 mg. portions), which was used principally for preparing the enzyme used in the present studies, were negative for the deaminase.

[3] The precipitate with this reagent is not obtained in consequence of its properties as a protein precipitant but because it gives the required pH for precipitation. Adjustment to the same pH with HCl will give a precipitate also.

The results obtained with a commercial sample of adenosine are somewhat low but this may be due to the presence of N-containing compounds other than adenosine in the sample. An experiment performed at pH 7.62, as was the experiment for Fig. 1, would also measure adenylic acid quantitatively. Adenosine triphosphate would be expected to be measured also (11). Adenosine can be estimated separately by performing the reaction at pH 5.9 at which the deaminase is still quite active but the phosphoesterase is inactive. The amount of enzyme used above completely deaminated 1.0 mg. of adenosine in 25 minutes at pH 5.9, but adenylic acid was not touched.

SUMMARY

An enzyme preparation from calf intestinal mucosa is described which deaminates adenosine and desoxyriboadenosine. The activity of the deaminase is conveniently measured manometrically; the use of the enzyme for estimating adenosine is suggested. The enzyme preparation contains phosphoesterases but these were inactive at pH 5.9, whereas the deaminase has considerable activity. The activity of the deaminase with adenosine as the substrate has a broad pH optimum at 7.0; the deaminase is very sensitive to silver, 4.0×10^{-7} moles per liter causing 50 per cent inhibition.

BIBLIOGRAPHY

1. Zittle, C. A., *J. Franklin Inst.*, **241**, 379 (1946).
2. Zittle, C. A., *J. Biol. Chem.*, **166**, 491 (1946).
3. Brady, T., *Biochem. J.*, **36**, 478 (1942).
4. Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, **70**, 243 (1926).
5. Schmidt, G., *Z. physiol. Chem.*, **179**, 243 (1928).
6. Schmidt, G., *Z. physiol. Chem.*, **208**, 185 (1932).
7. Conway, E. J., and Cooke, R., *Biochem. J.*, **33**, 479 (1939).
8. Klein, W., *Z. physiol. Chem.*, **255**, 82 (1938).
9. Brady, T. G., *Biochem. J.*, **35**, 855 (1941).
10. Sumner, J. B., and Myrbäck, K., *Z. physiol. Chem.*, **189**, 218 (1930).
11. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).

A MICROMETHOD FOR THE QUANTITATIVE PARTITION OF PHOSPHOLIPIDE MIXTURES INTO MONOAMINO-PHOSPHATIDES AND SPHINGOMYELIN*

By GERHARD SCHMIDT, JOSEPH BENOTTI, BESSIE HERSHMAN, AND S. J. THANNHAUSER

(From the Boston Dispensary, the Joseph H. Pratt Diagnostic Hospital, and Tufts College Medical School, Boston)

Thudichum (1) has already reported that sphingomyelin is much more resistant towards alkali than are the monophosphatides. Similar observations were made in this laboratory during a study involving the isolation of pure sphingomyelin from various organs. The difference between sphingomyelin and the monophosphatides in regard to their behavior towards alkali was found to be so striking that we were encouraged to search for conditions permitting the application of this difference as a basis for the quantitative partition of phospholipide mixtures. It was found that lecithin and cephalin are quantitatively transformed into acid-soluble P compounds by N potassium hydroxide at 37° within 24 hours. Under the same conditions sphingomyelin forms no acid-soluble P compounds.

In this paper we will describe a micromethod for the quantitative partition of phospholipide mixtures into monophosphatides and sphingomyelin; the underlying principle of this method is the selective saponification of the monophosphatides.

It appears that this method has considerable advantages in comparison with the current procedures based on the difference between the solubilities of the individual phospholipides (2) or their reineckates (3, 4). It is known that the solubilities of the phospholipides are very much influenced by the composition of the lipide mixture and by the presence of other substances such as electrolytes (5). These factors do not interfere with the saponification of the phospholipides. Another advantage of the "saponification method" is the possibility of detecting small amounts of monophosphatides in sphingomyelin samples. This is not possible with any of the current procedures, owing to the fact that the monophosphatides adhering to the sphingomyelin fractions are saturated lecithins which differ from unsaturated lecithins by the close resemblance of their solubility properties to those of sphingomyelin. Whereas it was formerly believed that hydrolecithins are not constituents of natural phospholipide mixtures,

recent observations have established their ubiquitous occurrence in animal tissues.   Lesuk and Anderson (6) isolated dipalmitolecithin from the lipides of the larvae of *Cysticercus fasciolaris*.   Thannhauser, Benotti, and Boncoddo[1] succeeded in isolating pure hydrolecithin from lung and spleen. The presence of hydrolecithin in brain has been made probable by observations of Merz (7) and of Thannhauser and his coworkers.[1]

Procedure

0.5 to 3 gm. of the finely ground tissue are extracted with 20 volumes of a boiling mixture of 3 parts of alcohol and 1 part of ether and then refluxed for 30 minutes with a mixture of equal volumes of chloroform and methanol. The extracts are pooled and evaporated to dryness under reduced pressure.[2] The residue is extracted with 5 cc. of the boiling chloroform-methanol mixture and brought to a volume of 10 cc. with the solvent.   An aliquot of 1 or 2 cc. is set aside for the determination of the total phosphorus.   Another suitable aliquot (1 or 2 cc.) is evaporated to dryness in a wide test-tube on the water bath after the addition of a few pieces of capillaries to prevent bumping.   The residue is dissolved in 0.2 cc. of boiling alcohol and emulsified in 5 cc. of N potassium hydroxide by blowing the alkali from a pipette vigorously into the alcoholic lipide solution.   The emulsion is shaken at 37° for 24 hours.   It is then precipitated by adding 1 cc. of 5 N hydrochloric acid and 5 cc. of 10 per cent trichloroacetic acid.   After standing for 2 hours (in order to hydrolyze the plasmalogens (8)) at room temperature the emulsion is centrifuged and filtered without suction.   A water-clear filtrate is usually obtained.   If, in rare instances, the filtrates remain opalescent, the saponification is repeated with a new aliquot.   Before the acidification, 1 cc. of a 1 per cent solution of egg albumin is added to the emulsion.   The procedure is then continued in the usual manner.

Determinations of the total phosphorus are carried out in an aliquot of the original lipide extract set aside for this purpose and in an aliquot (8 cc.) of the acid filtrate obtained after saponification.   The amount of the total P in the original lipide extract corresponds to the total phospholipide P, the difference between this value and that of the saponifiable P (determined as

---

[1] Thannhauser, S. J., Benotti, J., and Boncoddo, N. F., unpublished experiments.

[2] The evaporation of the alcohol-ether extract to dryness gives very satisfactory results with all fresh tissues.   For lipide determinations in autolyzed organs (especially pancreas and small intestines) it is advisable to concentrate the alcohol-ether extract to a volume of 15 to 20 cc.   At this stage the protein breakdown products, carbohydrates and other contaminating non-lipides, are precipitated by adding an excess of petroleum ether.   The filtrate is then evaporated to dryness.   After re-extraction with chloroform-methanol the procedure is continued as described above.

total P in the trichloroacetic acid filtrate after saponification with N KOH) to the amount of the sphingomyelin P.

Preparation of Phospholipides—Lecithin was prepared according to Levene and Rolf (9) from eggs or brain; the samples of cephalin used were obtained according to the directions of Folch (10) for the preparation of mixtures of phosphatidyl ethanolamine, serine cephalin, and inositol phosphatide.[3] Pure sphingomyelin was isolated from lung according to a new procedure developed by Thannhauser, Benotti, and Boncoddo.[1] We wish to emphasize that in this procedure any treatment with alkali is avoided. The sphingomyelin was identified by its physical properties and by its nitrogen and phosphorus content. The absence of monophosphatides was ascertained by microdeterminations of glycerol according to Blix (11), the absence of cerebrosides by the negative result of the Molisch test.

Behavior of Individual Phospholipides and Phospholipide Mixtures of Known Composition Against N *Potassium Hydroxide under Mild Conditions*— Suitable aliquots of solutions of the phosphatides were pipetted into wide Pyrex test-tubes and brought to dryness on a water bath. Each sample was emulsified with 5 cc. of N potassium hydroxide and slowly shaken at 37° for 24 hours. The emulsion was precipitated by the addition of 1 cc. of 5 N hydrochloric acid and 5 cc. of 10 per cent trichloroacetic acid. After standing 1 hour at room temperature[4] the samples were spun in an angle centrifuge and filtered. The amounts of total phosphorus of the original phospholipide solutions and those of the clear trichloroacetic acid filtrates were determined according to the method of Fiske and Subbarow (12). The results of a typical experiment are recorded on Table I.

The conclusions drawn from the preceding experiments, namely the absence in the saponifiable P fraction of P compounds originating from sphingomyelin, could be substantiated in experiments in which an entirely different method of analysis was employed. In these experiments use was made of the fact that the treatment of the monophosphatides with alkali results not only in the hydrolysis of the fatty acid groups, but also in the

[3] The cephalin was reprecipitated twice with hot alcohol from its concentrated ethereal solution in order to remove sphingomyelin. Crude cephalin is always contaminated with sphingomyelin which is insoluble in cold alcohol but easily soluble in hot alcohol.

[4] The standing of the acidified mixture has the purpose of destroying any plasmalogen which might be present. Plasmalogen is very resistant against alkali, but it is quickly hydrolyzed by acids at room temperature (8).

almost complete hydrolysis of the ester linkages between the phosphorus and nitrogen groups. Thus the acid-soluble P fraction obtained after the alkaline saponification of monophosphatides consists of monoesters of phosphoric acids which contain only negligible amounts of nitrogen. We were able to demonstrate that the acid-soluble P fraction obtained after the saponification of mixtures of monophosphatides and sphingomyelin such as the ether-insoluble lipides of brain (protagon of the older literature (13)) contained only very small amounts of nitrogen. (The equivalent ratio of N:P was 1:14.[5]) Since any organic P compound originating from the hydrolysis of sphingomyelin has an N:P ratio of 1:1 or 2:1, this finding excludes the presence of more than negligible quantities of sphingomyelin

TABLE I

Effect of Mild Treatment with Alkali on Phosphatides and Phosphatide Mixtures

Phosphatide	Total P	Acid-soluble P after alkali treatment
	mg.	*mg.*
Lecithin	0.273	0.279
Cephalin	0.318	0.310
Sphingomyelin	0.339	0.015
Mixture of lecithin	0.500	
+ cephalin	0.316 (0.816)*	0.801
Mixture of lecithin	0.273	
+ cephalin	0.500 (0.773)*	0.773
+ sphingomyelin	0.339	

* Sum of lecithin and cephalin.

P in the saponifiable phosphorus fraction obtained from brain protagon. (The protagon used in these experiments contained sphingomyelin and monophosphatides in approximately equal amounts.)

[5] The experiments were carried out on batches of approximately 30 gm. of protagon. The acid-soluble P fraction was quantitatively isolated as a mixture of barium salts by alcohol precipitation. No inorganic P was formed during the saponification. The small amount of nitrogen which was found in the acid-soluble P fraction obtained after saponification of the phospholipides is most likely due to the presence of colamine phosphoric acid diesters which are more resistant to alkali than the corresponding choline phosphoric acid diesters. This assumption is supported by the observation that 85 per cent of the total nitrogen of the saponifiable P fraction was amino nitrogen. Furthermore, after the refluxing of the barium salts with saturated aqueous barium hydroxide, the nitrogen was quantitatively found in the supernatant. This finding renders it very unlikely that the nitrogen originated from sphingosine, which is insoluble in water. Even under the most unfavorable assumption that the nitrogen of the saponifiable P fraction would be entirely present in the form of sphingosine, it would account for only 7 per cent of the sphingomyelin of the lipide mixture used in the experiment.

TABLE II

Amounts of Monophosphatides and Sphingomyelin in Various Tissues

Animal	Tissue	P per 100 gm. fresh tissue		Sphingomyelin P × 100 / Total lipide P			
		Mono-phos-phatides (3)	Sphin-gomyelin (4)	Computed from (3) and (4)	Williams et al. (14)	Artom (15)	Thann-hauser et al. (3)
(1)	(2)						
		mg.	*mg.*				
Rat	Brain	211	13	5.8			
		194	7	3.5			
		250	12	4.6			
		191	12	5.9	15.7		
"	Sciatic nerve	256	74	22.5			
		276	50	15.3			
		168	80	32.2			
		284	76	21.2			
		315	65	17.1			
Cat	Brain	173	55	24.4			
"	Sciatic nerve	156	146	50.0			
		192	153	44.5			
		184	148	44.5			
Beef	Brain gray matter	158	12	7.6			
"	" white "	295	93	31.2			
Rat	Liver	112	3	2.6			
		165	4	2.5			
		138.5	0	0			
		153	3	2.0			
		147	0	0			
		157	0	0	2.5		
"	Kidney	123	20	14.0			
		118	22	15.7			
		117	18	13.3	12.1		
"	Lung	75.3	11.9	13.6			
		77.7	12.6	14.0	18.8		
"	Spleen	81.2	13.3	14.1			
		72.3	9.7	11.0			
		89.0	19.0	17.5	10.3		
"	Heart	84.5	4.3	4.9			
		87.0	5.0	5.4			
		91.5	5.2	5.4	3.1		
Beef	Pancreas	116	6.0	4.9			
		98	2.0	2.0			
		93.5	4.5	4.6			
Human	Plasma	8.6	1.8	17.5			
		8.8	1.1	11.0		12	10.2

Amounts of Monophosphatides and Sphingomyelin in Some Tissues—
Table II shows representative figures of the content of monophosphatides
and sphingomyelin in some animal organs.   Table II also contains average

figures obtained for sphingomyelin by Williams and his associates (14), by Artom (15), and by Thannhauser and his coworkers (3). In these investigations the reineckate method developed by Thannhauser, Benotti, and Reinstein (3) or modifications of this method were used.

DISCUSSION

Since the amounts of monophosphatides are always larger than those of sphingomyelin, the method permits the accurate determination of this fraction in all tissues. On the other hand, the sphingomyelin content of some tissues (liver, heart) is so small that the figures of sphingomyelin can only be considered as approximate values in these cases, since the amounts of sphingomyelin are obtained by difference.

The errors caused by the possible presence of non-lipide P compounds in the final lipide extracts are comparatively small according to a recent investigation by Gortner (16). Since all contaminating non-lipide P compounds are acid-soluble, these errors would affect only the values of the monophosphatides but not those of sphingomyelin.

Apart from its application to tissue analysis, the procedure represents a sensitive test for the presence of monoaminophosphatides in samples of sphingomyelin.

SUMMARY

1. A method for the quantitative microdetermination of sphingomyelin in lipide mixtures based on the selective saponification of the monophosphatides has been described.

2. It was found that the lipides of the sciatic nerve of the cat and the rat contain a much higher percentage of sphingomyelin than those of the brain. The lipide fraction of the white matter of beef brain contains a much higher percentage of sphingomyelin than that of the cortex.

BIBLIOGRAPHY

1. Thudichum, J. L. W., Die chemische Konstitution des Gehirns des Menschen und der Tiere, Tübingen, 170 (1901).
2. Kirk, E., *J. Biol. Chem.*, **123**, 623 (1938).
3. Thannhauser, S. J., Benotti, J., and Reinstein, H., *J. Biol. Chem.*, **129**, 709 (1939).
4. Erickson, B. N., Arvin, I., Teague, D. M., and Williams, H. H., *J. Biol. Chem.*, **135**, 671 (1940).
5. Sinclair, R. G., and Dolan, M., *J. Biol. Chem.*, **142**, 659 (1942).
6. Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **139**, 457 (1941).
7. Merz, W., *Z. physiol. Chem.*, **196**, 10 (1931).
8. Feulgen, R., and Bersin, T., *Z. physiol. Chem.*, **260**, 217 (1939).
9. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **72**, 587 (1927).
10. Folch, J., *J. Biol. Chem.*, **146**, 35 (1942).

11. Blix, G., *Mikrochim. acta*, **1,** 75 (1937).
12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
13. Liebreich, O., *Ann. Chem.*, **134,** 29 (1865).
14. Williams, H. H., Galbraith, H., Kaucher, M., Moyer, E. Z., Richards, A. J., and Macy, I. G., *J. Biol. Chem.*, **161,** 475 (1945).
15. Artom, C., *J. Biol. Chem.*, **157,** 595 (1945).
16. Gortner, W. A., *J. Biol. Chem.*, **159,** 97 (1945).

THE UTILIZATION OF d-AMINO ACIDS BY MAN*

VI. TYROSINE

By ANTHONY A. ALBANESE, VIRGINIA IRBY, AND MARILYN LEIN

(From the Department of Pediatrics, New York University College of Medicine, and the Children's Medical Service, Bellevue Hospital, New York)

The growth experiments of Womack and Rose (1) and the studies of Moss and Schoenheimer (2) have established the conversion of phenlyalanine to tyrosine in the rat. Recent studies of Rose and associates (3) indicate that this biochemical reaction also occurs in man. The reverse reaction, the conversion of tyrosine into phenylalanine, has been found not to proceed at a rate commensurate with the growth needs of the rat (1) and remains to be tested in man. Despite the apparent dietary dispensability and ready commercial availability of *l*-tyrosine, we felt that information concerning the metabolism of the unnatural variety would be of interest in view of the physiological indispensability of the *l* isomer and known pharmacodynamic differences of the optical isomers of its biochemically related substances, *e.g.* thyroxine and epinephrine.

The determination of the degree of utilization of the unnatural amino acids in man by the excretion procedure which we have previously described (4) seemed uniquely suited in this instance in which growth (1) and nitrogen equilibrium (3) measurements would not be effective because of the dietary dispensability of tyrosine for these body functions (1, 3). The data of four experiments disclosed that within the 7 hour period succeeding oral administration of 0.01 mole (1.82 gm.) of *dl*-tyrosine 134 ± 19 mg. more tyrosine was excreted in the urine than after the feeding of 0.01 mole of *l*-tyrosine. This increase in tyrosine which is equivalent to 14.5 ± 2.5 per cent of the *d* form fed and the increase in total organic acid output which appears to arise from a metabolic degradation of the unexcreted *d*-tyrosine fraction of the racemate fed suggest that this tyrosine isomer is not available to the human for normal physiological purposes.

EXPERIMENTAL

Preparation of dl-Tyrosine—Racemic tyrosine was prepared by acid hydrolysis of diacetyl-*dl*-tyrosine synthesized according to the method of du Vigneaud and Meyer (5) from *l*(−)-tyrosine (Merck), containing

7.60 per cent N by micro-Kjeldahl analysis and having a specific rotation of $[\alpha]_D^{20}$ (4.04 gm. per 100 cc. of 1 N HCl) = $-9.82°$. After two recrystallizations, 45 gm. of *l*-tyrosine yielded 20 gm. of a product of zero optical rotation in 1 N HCl, which contained 7.40 per cent N.

Colorimetric Estimation of Tyrosine in Human Urine—Attempts to adapt available methods for the estimation of tyrosine in protein digests to the determination of urinary tyrosine indicated the procedure of Lugg (6) to be best suited to this purpose. Recovery tests with urine specimens submitted to continuous ether extraction for 24 hours in a small all-glass modified Schacherl extraction apparatus[1] and protein hydrolysates demonstrated that the technique is adequate for analytical work.

Reagents—

Precipitation reagent. 75 gm. of $HgSO_4$, 55 gm. of $HgCl_2$, and 70 gm. of Na_2SO_4 were dissolved in a mixture of 850 cc. of water and 68 cc. of concentrated H_2SO_4; the solution was then made to 1 liter.

Washing reagent. 500 cc. of precipitation reagent were diluted to 1 liter with 1 N H_2SO_4.

Sulfuric acid, 7 N solution.

Sodium nitrite, 0.8 per cent solution. This should be stored in the refrigerator and a fresh solution should be prepared every 2 weeks.

Ether. Commercially available reagent grades are satisfactory.

Tyrosine standard. 40 mg. of *l*-tyrosine (Merck) were dissolved in 100 cc. of 1 N H_2SO_4.

Procedure—Aliquots of protein hydrolysates or urine (5 to 10 cc.) containing 0.2 to 1.0 mg. of tyrosine were transferred by pipette to 40 cc. graduated conical bottom centrifuge tubes and diluted to 20 cc. volume. After the addition of 5 cc. of precipitation reagent, the tubes were immersed in a boiling water bath for 10 minutes and 4 cc. of 7 N sulfuric acid added subsequent to cooling the reaction mixtures in an ice bath. The volume of the cooled samples was adjusted to 30 cc. with water and 20 to 30 mg. of Celite (Johns-Manville) were added with vigorous stirring. The precipitates were collected by centrifugation and decantation of the supernatant liquid and were then washed by a repetition of this procedure after resuspension of the precipitate in 10 cc. of the washing reagent. Both the original and washing supernatant fluids were collected in 50 cc. volumetric flasks and made to volume subsequent to the addition of 1 cc. of sodium nitrite solution. We have found it unnecessary to heat the samples at this point as recommended by Lugg. After standing for 5 minutes, the resulting color was read in the Klett-Summerson photoelectric colorimeter with Filter S-52 and compared with that of a similarly treated tyrosine standard

[1] This apparatus, catalogue No. 1500, was obtained from Eck and Krebs, 131 West 24th Street, New York.

(1 cc.). The color persists for about 45 minutes and the reagent blank should give a zero reading at all times.

Results—The data obtained by submitting varying amounts of the tyrosine standard to the color reaction give evidence that the reaction conforms to Beer's law (Table I). Application of the procedure to acid and enzymatic digests of various proteins yielded tyrosine values which, except for gelatin, are in fair agreement with those recorded in the literature (7).

TABLE I

Relation of Color Intensity to Amount of Tyrosine

$l(-)$-Tyrosine	Klett readings*
mg.	
0.2	50
0.4	98
0.8	199
1.0	248
1.4	350
1.6	400
1.8	450

* Readings were made in a Klett-Summerson photoelectric colorimeter with Filter S-52 and each value represents the average of five duplicate determinations.

TABLE II

Recovery of Added Tyrosine from Mixtures

Sample	Tyrosine added	Tyrosine found	Recovery of added tyrosine
	mg.	*mg.*	*per cent*
Urine, 1 cc..................................		0.145	
" 1 "	0.772	0.910	99.1
l-Tryptophane, 2 mg......................	0.772	0.770	99.7
Urea, 10 mg...............................	0.386	0.389	100.3
Sodium chloride, 10 mg...................	0.386	0.385	99.9
Ammonium sulfate, 10 mg................	0.386	0.383	99.6
Casein hydrolysate			
1 mg. total N...........................		0.408	
1 " " "	0.386	0.794	100.0

The tyrosine figure for gelatin, 0.32 per cent tyrosine nitrogen of total N, is reported here merely to indicate the quantitative variability which may exist in this product. The favorable results obtained with recovery tests on 24 hour ether-extracted urine specimens and experiments on the addition of urinary metabolites to 1 cc. aliquots of the standard which might interfere with the modified Millon's reaction point to the suitability of the procedure for the estimation of urinary tyrosine (Table II). Analyses of

ether-extracted 24 hour urine specimens from twelve adult males (50 to 110 kilos) on normal diets and from six normal infants (10 to 12 kilos) on diluted cow's milk formulae showed the daily tyrosine output to be 213 ± 19.9 mg. and 28.7 ± 6.5 mg. respectively. This represents an excretion of about 3.0 mg. of tyrosine per kilo of body weight irrespective of the individual ages.

Human Experiment

Subsequent to the elimination of the control urine specimen which was collected 2 hours after a light breakfast, the subjects were given by mouth
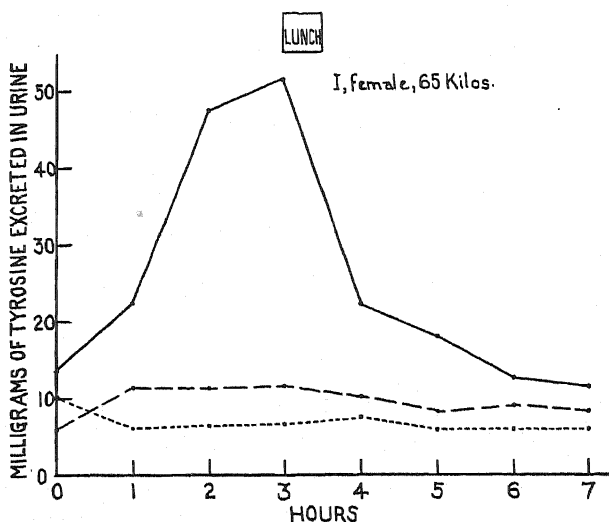
FIG. 1. Urinary output of subject I, following ingestion of 0.01 mole (1.82 gm.) of $l(-)$- and dl-tyrosine. The solid line represents excretion after administration of the racemate, the broken line after feeding the l isomer, and the dotted line normal output.

0.01 mole (1.82 gm.) of $l(-)$- or dl-tyrosine suspended in 240 cc. of water, and an additional 120 cc. of water were also given at the end of each of the succeeding 6 hours. The urines were collected hourly and analyzed immediately for total organic acids (8), ether-soluble free phenols (9), and tyrosine as described below. Since the ingestion of food did not seem to influence the output of these metabolites, the fast was maintained only for the first 3 or 4 hours of these experiments. Control experiments, in which no tyrosine was fed, were similarly performed.

Tyrosine excretion curves of one of the experiments are shown in Fig. 1 and indicate that, whereas the administration of l-tyrosine causes only a

slightly increased output of tyrosine above the normal, the ingestion of the racemate results in a transient but marked elevation of urinary tyrosine. It is to be noted that the tyrosine levels of the urine returned to the normal in all experiments within 5 to 6 hours after *dl*-tyrosine was given.

TABLE III

Total Urinary Tyrosine for 7 Hour Period Following Administration of 0.01 Mole of l- or dl-Tyrosine

1.82 gm. of *dl*-tyrosine ≈ 0.91 gm. of *d*(+)-tyrosine.

| Subject | Normal tyrosine output | Tyrosine output after administration of isomers | | (b) − (a) | Recovery in urine of d form fed |
		l (a)	dl (b)		
	mg.	mg.	mg.	mg.	per cent
A, ♂, 70 kilos	27	39	180	141	15.7
" ♂, 70 "	33	46	210	164	18.0
L, ♀, 60 "	30	61	169	108	12.0
I, ♀, 65 "	30	69	190	121	12.5

TABLE IV

Organic Acid and Free Phenol Output for 7 Hour Period Following Administration of 0.01 Mole of l- or dl-Tyrosine*

| Subject | Normal output | | Output after administration of tyrosine isomers | | | | (b) − (a) | | d-Tyrosine not excreted in urine | Molar ratio, organic acids to unexcreted d-tyrosine |
| | | | l (a) | | dl (b) | | | | | |
	Organic acids	Free phenols	Organic acids	Free phenols	Organic acids	Free phenols	Organic acids	Free phenols		
	m.eq. HCl	mg.	m.eq. HCl	mg.	m.eq. HCl	mg.	m.eq. HCl	mg.	m.eq.	
I, ♀, 65 kilos.......	29.2	205	29.7	264	48.0	262	+18.3	−2	4.42	4.14
L, ♀, 60 "	18.8	205	18.2	203	34.5	238	+16.3	+35	4.35	3.75
A, ♂, 70 "	19.8	153	20.4	158	36.8	168	+16.4	+10	4.10	4.00

* All organic acid values corrected for tyrosine content of the urine; 1 milliequivalent of tyrosine was found by titrations to be equivalent to 1 milliequivalent of organic acid.

The amount of urinary tyrosine arising from the ingestion of the *d* variety can be ascertained from the difference in tyrosine output for the 7 hour period following administration of racemic and naturally occurring tyrosine. These calculated values are listed under the heading "(b) − (a)" in Table III and disclose that the feeding of 0.01 mole of *dl*-tyrosine produced an increment in tyrosine output equivalent to 12.0 to 18.0 per cent of the *d* component.

Since, according to either the Dakin concept (10) or the Neubauer theory (11), the intermediary metabolism of tyrosine involves a fission of the benzene ring and subsequent formation of ketonic fatty acids, it was felt that measurement of the total organic acids in our experiments might give additional information on the metabolic fate of $d(+)$-tyrosine. These determinations were done and disclosed that the ingestion of dl-tyrosine, unlike $l(-)$-tyrosine, causes an elevation in organic acid output above normal levels. Presuming that this organic acid excess arises from the unexcreted fraction of $d(+)$-tyrosine fed in the racemate, we have calculated the molar ratios of organic acids to the moiety of ingested $d(+)$-tyrosine not accounted for as tyrosine in the urine and found these to approximate the whole number 4.0. The acid equivalence of this tyrosine fraction and similarity in output of ether-extractable free phenols (Table IV) subsequent to administration of $l(-)$- and dl-tyrosine would seem to preclude the aromatic nature of the excreted organic acids and point to the metabolic degradation of d-tyrosine to the aliphatic acid stage. Since this value also represents the maximum amount of acid which could be derived from tyrosine by the Dakin or Neubauer schemes, it seems reasonable to assume that, except for the portion excreted as tyrosine, nearly all of the orally administered d-tyrosine is degraded to organic acids and very little if any becomes available to the body for normal physiological functions.

Comments

The ability of the rat to grow and of man to continue in N balance in the absence of dietary tyrosine does not permit measurements of the availability of $d(+)$-tyrosine by these criteria. *In vitro* studies, however, have indicated that both optical isomers of tyrosine are degraded to the same extent and manner by various animal tissues (12). Our findings, the increased output of tyrosine and aliphatic organic acids which together would seem to account for nearly all of the $d(+)$-tyrosine fed in the racemate, suggest that unnatural tyrosine is so metabolized by man as not to be available for normal biochemical functions. The non-aromatic nature of the organic acids excreted as a result of the ingestion of $d(+)$-tyrosine precludes their identity with homogentisic acid or p-hydroxyphenylpyruvic acid and points to the difference of this metabolic failure of $d(+)$-tyrosine from that of $l(-)$-tyrosine in human vitamin C deficiency (13).

The observation of Cushny (14) that natural l-epinephrine is nearly twice as strong a vasoconstrictor as the racemate led him to conclude that the dextrorotatory base was nearly devoid of action on the sympathetic terminations in the arterioles. Foster, Palmer, and Leland (15) observed that l-thyroxine has a calorigenic effect on normal guinea pigs approximately twice as great as that of racemic thyroxine. These previous

investigations and our present report suggest that mammalian organisms are not able to metabolize d-hydroxyphenolic substances in the same manner as the l variety.

SUMMARY

It has been found that within 7 hours after the oral administration of dl-tyrosine to adult humans an excess of urinary tyrosine and aliphatic organic acids is excreted which would seem to account for nearly all of the d component fed. These findings are interpreted to indicate that $d(+)$-tyrosine unlike $l(-)$-tyrosine is metabolized in man in such a manner as to render it unavailable for normal physiological functions.

BIBLIOGRAPHY

1. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **107**, 449 (1934).
2. Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 415 (1940).
3. Rose, W. C., Haines, W. J., and Johnson, J. E., *J. Biol. Chem.*, **146**, 683 (1942).
4. Albanese, A. A., *Bull. Johns Hopkins Hosp.*, **75**, 175 (1944).
5. du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98**, 295 (1932).
6. Lugg, J. W. H., *Biochem. J.*, **31**, 1423 (1937).
7. Block, R. J., and Bolling, D., The amino acid compositon of proteins and foods, Springfield, 112–141 (1945).
8. Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, **41**, 567 (1920).
9. Marenzi, A. D., *Compt. rend. Soc. biol.*, **107**, 374 (1931). Schmidt, E. G., *J. Biol. Chem.*, **145**, 533 (1942).
10. Dakin, H. D., Oxidations and reductions in the animal body, Monographs on biochemistry, London, 84 ff. (1922).
11. Neubauer, O., *Deut. Arch. klin. Med.*, **95**, 211 (1909).
12. Felix, K., and Di Marco, I., *Kolloid Z.*, **89**, 293 (1939). Edson, N. L., *Biochem. J.*, **29**, 2498 (1935).
13. Levine, S. Z., Marples, E., and Gordon, H. H., *J. Clin. Invest.*, **20**, 199 (1941). Levine, S. Z., Gordon, H. H., and Marples, E., *J. Clin. Invest.*, **20**, 209 (1941).
14. Cushny, A. R., Biological relations of optically isomeric substances, Baltimore, 46–47 (1926).
15. Foster, G. L., Palmer, W. W., and Leland, J. P., *J. Biol. Chem.*, **115**, 467 (1936).

CYSTEINE LOSSES RESULTING FROM ACID HYDROLYSIS OF PROTEINS

By MURRAY HALWER and GEORGE C. NUTTING

(*From the Eastern Regional Research Laboratory,* * *Philadelphia*)

In the course of a study of the relation between molecular cross-bonding and the dimensional stability of fibrous proteins, particularly the keratins, it was necessary to determine small amounts of cysteine, about 1 per cent of the sample. For this purpose, a method used by a number of workers was adopted. This method consisted in hydrolysis of the protein by refluxing it with acid, after which the cysteine in the hydrolysate was determined by oxidation with a selective oxidizing agent, phosphotungstic acid being used in our experiments. Control experiments showed, however, that when small amounts of cysteine, comparable to the amounts which the proteins were thought to contain, were added to the intact samples and the mixtures were subjected to digestion with the acid the added cysteine was only incompletely recovered. In one experiment in which the digestion was carried out in the presence of air, as some workers have done, the added cysteine vanished entirely.

The object of this paper is to demonstrate the inadequacy of the acid hydrolysis method for the determination of cysteine, and to report on studies of some of the factors responsible for the incomplete recovery.

EXPERIMENTAL

Except where otherwise noted, 100 mg. of protein (± 10 per cent) were weighed out for the digestion. To this were added either 1, 2.5, or 5 mg. of cysteine (all ± 10 per cent) as the hydrochloride. Two methods of hydrolysis were used. (1) The mixture of protein and added cysteine, contained in 2 ml. of 6 N HCl, was placed in a test-tube. To remove air, the tube was evacuated, then flushed with carbon dioxide which had been freed of oxygen by passing it over heated copper. This procedure was repeated several times. Finally, the tube was sealed off while evacuated and heated for 16 to 20 hours at 115°. (2) To the mixture of protein and added cysteine, contained in a 100 ml. acetylation flask, were added enough water to make the total amount of water 5 ml., 11 ml. of concentrated HCl, and 2 gm. of urea. The flask was attached to a reflux condenser and the system evacuated. Oxygen-free carbon dioxide was now admitted, and the pro-

---

* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

cedure repeated several times. A slow stream of carbon dioxide was allowed to pass through the system while the flask was heated in an oil bath to the point of gentle refluxing. The heating was continued for 6 hours.

This second procedure is essentially that of Brand and Kassell (1). Its purpose is to minimize formation of humin, an objective which they considered desirable in view of Lugg's (2) observation that cysteine reacts with humin. We have found it to be highly effective in preventing formation of insoluble matter. It gives perfectly clear and nearly colorless hydrolysates with horse serum albumin, ovalbumin, and lactoglobulin; the hydrolysate given by wool is faintly cloudy.

The acid was removed by vacuum distillation in a carbon dioxide atmosphere, and the residues were taken up in water and diluted to volume. The hydrolysates obtained by the sealed tube method were diluted to volume directly.

Cysteine was determined by the modified phosphotungstic acid method of Kassell and Brand (3).

Materials

Horse serum albumin[1] was prepared from lyophilized horse serum by the method of Adair and Robinson (4). It was recrystallized three times.

Ovalbumin was prepared from the whites of fresh eggs by the method of Sørensen and Høyrup (5). It was not recrystallized.

Lactoglobulin[1] was prepared from fresh milk by the method of Sørensen and Sørensen (6). It was recrystallized once.

The wool sample[1] had been scoured by the manufacturer in hot soap solution. It was further purified by extracting it successively with alcohol, ether, and water at room temperature.

Black human hair was purified by successive extraction with alcohol, ether, and water at room temperature.

Cysteine hydrochloride was obtained from the Eastman Kodak Company. Recrystallization did not affect its reducing power toward phosphotungstic acid.

Results

Table I shows the recoveries observed when cysteine was added to the proteins named above and the mixtures were submitted to the hydrolysis procedures outlined. It should be noted that these are "uncorrected" recoveries. They were calculated by subtracting, from the total amount of cysteine found, the amount contributed by the protein itself, as determined

[1] We should like to thank Sharp and Dohme, Inc., for supplying the lyophilized horse serum; Dr. W. G. Gordon of this Laboratory for lactoglobulin; Dr. Werner von Bergen, Forstmann Woolen Company, for the wool.

by a separate experiment in which no cysteine was added. This procedure can give accurate results only if the fraction of the cysteine lost is independent of the amount of cysteine present. Table I shows, however, that in all probability this is not the case, since the uncorrected recovery values show a strong dependence on the amount of cysteine, with 5 mg. additions giving much higher recoveries than the 1 mg. additions. It is

TABLE I

Recovery of Cysteine in Acid Digestion of Mixture of Proteins and Added Cysteine*

Experiment No.	Protein	Cysteine added	Hydrolysis method	Per cent recovery of added cysteine†
		mg.		
1	Wool	1	Sealed tube	42.2
2	"	2.5	" "	57.5
3	"	5	" "	62.0
4	"	1	Urea	51.2
5	"	5	"	72.0
6	Human hair	1	Sealed tube	40.2
7	Ovalbumin	1	" "	59.0
8	"	2.5	" "	79.2
9	"	5	" "	63.5
10	Horse serum albumin	1	" "	31.9
11	" " "	2.5	" "	55.5
12	" " "	5	" "	70.0
13	" " "	1	Urea	33.3
14	" " "	5	"	73.5
15	Lactoglobulin	5	Sealed tube	81.6
16	"	5	Urea	79.2
17	None	5	Sealed tube	99.3
18	"	5	Urea	97.5

* The protein sample was approximately 100 mg. in each case. The terms "sealed tube" and "urea" refer, respectively, to the first and second methods of hydrolysis discussed under the heading "Experimental." The recovery value for the sealed tube control, Experiment 17, is the average of three, the individual values being 99.0, 99.4, 99.4 per cent. The recovery value for the urea control, Experiment 18, is the average of two, the individual values being 97.4 and 97.5 per cent.

† These are uncorrected recoveries; see the text.

therefore to be expected that the contribution of the protein itself will be larger in the recovery experiments than the values obtained in the absence of added cysteine. The recovery figures given in Table I are therefore probably too high.

The values found for the cysteine contents of these proteins, in the absence of added cysteine, are given in Table II. These, too, are "uncorrected," since it is not possible from the data at hand to say what is the proper correction factor to apply to them.

cedure repeated several times. A slow stream of carbon dioxide was allowed to pass through the system while the flask was heated in an oil bath to the point of gentle refluxing. The heating was continued for 6 hours.

This second procedure is essentially that of Brand and Kassell (1). Its purpose is to minimize formation of humin, an objective which they considered desirable in view of Lugg's (2) observation that cysteine reacts with humin. We have found it to be highly effective in preventing formation of insoluble matter. It gives perfectly clear and nearly colorless hydrolysates with horse serum albumin, ovalbumin, and lactoglobulin; the hydrolysate given by wool is faintly cloudy.

The acid was removed by vacuum distillation in a carbon dioxide atmosphere, and the residues were taken up in water and diluted to volume. The hydrolysates obtained by the sealed tube method were diluted to volume directly.

Cysteine was determined by the modified phosphotungstic acid method of Kassell and Brand (3).

Materials

Horse serum albumin[1] was prepared from lyophilized horse serum by the method of Adair and Robinson (4). It was recrystallized three times.

Ovalbumin was prepared from the whites of fresh eggs by the method of Sørensen and Høyrup (5). It was not recrystallized.

Lactoglobulin[1] was prepared from fresh milk by the method of Sørensen and Sørensen (6). It was recrystallized once.

The wool sample[1] had been scoured by the manufacturer in hot soap solution. It was further purified by extracting it successively with alcohol, ether, and water at room temperature.

Black human hair was purified by successive extraction with alcohol, ether, and water at room temperature.

Cysteine hydrochloride was obtained from the Eastman Kodak Company. Recrystallization did not affect its reducing power toward phosphotungstic acid.

Results

Table I shows the recoveries observed when cysteine was added to the proteins named above and the mixtures were submitted to the hydrolysis procedures outlined. It should be noted that these are "uncorrected" recoveries. They were calculated by subtracting, from the total amount of cysteine found, the amount contributed by the protein itself, as determined

by a separate experiment in which no cysteine was added. This procedure can give accurate results only if the fraction of the cysteine lost is independent of the amount of cysteine present. Table I shows, however, that in all probability this is not the case, since the uncorrected recovery values show a strong dependence on the amount of cysteine, with 5 mg. additions giving much higher recoveries than the 1 mg. additions. It is

TABLE I

Recovery of Cysteine in Acid Digestion of Mixture of Proteins and Added Cysteine*

Experiment No.	Protein	Cysteine added	Hydrolysis method	Per cent recovery of added cysteine†
		mg.		
1	Wool	1	Sealed tube	42.2
2	"	2.5	" "	57.5
3	"	5	" "	62.0
4	"	1	Urea	51.2
5	"	5	"	72.0
6	Human hair	1	Sealed tube	40.2
7	Ovalbumin	1	" "	59.0
8	"	2.5	" "	79.2
9	"	5	" "	63.5
10	Horse serum albumin	1	" "	31.9
11	" " "	2.5	" "	55.5
12	" " "	5	" "	70.0
13	" " "	1	Urea	33.3
14	" " "	5	"	73.5
15	Lactoglobulin	5	Sealed tube	81.6
16	"	5	Urea	79.2
17	None	5	Sealed tube	99.3
18	"	5	Urea	97.5

* The protein sample was approximately 100 mg. in each case. The terms "sealed tube" and "urea" refer, respectively, to the first and second methods of hydrolysis discussed under the heading "Experimental." The recovery value for the sealed tube control, Experiment 17, is the average of three, the individual values being 99.0, 99.4, 99.4 per cent. The recovery value for the urea control, Experiment 18, is the average of two, the individual values being 97.4 and 97.5 per cent.

† These are uncorrected recoveries; see the text.

therefore to be expected that the contribution of the protein itself will be larger in the recovery experiments than the values obtained in the absence of added cysteine. The recovery figures given in Table I are therefore probably too high.

The values found for the cysteine contents of these proteins, in the absence of added cysteine, are given in Table II. These, too, are "uncorrected," since it is not possible from the data at hand to say what is the proper correction factor to apply to them.

Examination of Table I shows that in no case could the added cysteine be completely recovered. The loss was particularly serious for the lower levels of added cysteine, although even at the 5 mg. level the loss was marked. Untreated proteins almost certainly contain much less than 5 per cent cysteine. Therefore, values obtained for these materials by acid hydrolysis methods may be appreciably low. Although the urea method is successful in preventing the formation of insoluble humin, Table I (Experiments 10 and 13, 12 and 14, 15 and 16) shows that it fails in three out of five cases to bring about any distinct improvement in cysteine recovery, compared with the sealed tube method. Moreover, Table II shows that it does not give higher values for the original cysteine content.

Two hypotheses suggest themselves to account for the missing cysteine: (1) It has reacted with residual oxygen, which has entered the system despite all precautions to exclude it; (2) it has reacted with some substance in

TABLE II

Cysteine Content of Proteins

Protein	Per cent cysteine*	
	Sealed tube method	Urea method
Wool.....................................	0.68	0.33
Human hair............................	0.42	
Ovalbumin.............................	0.71	
Horse serum albumin.................	0.39	0.32
Lactoglobulin.........................	1.01	1.01

* These values are on a moisture-free basis. They are uncorrected; see the text.

the protein sample, present either as an impurity or as part of the protein molecule.

The excellent recoveries of cysteine in the control experiments (Nos. 17 and 18, Table I) make the first hypothesis unlikely, since there was the same opportunity for oxygen to enter these systems as those containing protein. As a further check, a recovery experiment was performed on wool, identical with Experiment 5 in Table I, except that the quantities of wool and added cysteine were 5 times greater. If the loss of cysteine is due to reaction with a small amount of residual oxygen, then the fraction lost should be sharply decreased by increasing the amount of cysteine. Only a minor improvement in the recovery was observed, however, the value being 76.5 per cent.

The good recovery in the control samples also disposes of the possibility that the cysteine is decomposed in some fashion under the hydrolysis conditions, whether protein is present or not.

Concerning the second hypothesis, it is well known that cysteine can

react with reducing sugars and with aldehydes in general (see, for example, Schubert (7)). Many proteins contain firmly attached polysaccharide residues which are capable of producing simple sugars on digestion with acid. Many other protein preparations are likely to contain carbohydrate as an impurity. That glucose, at least, is capable of reacting with cysteine under the conditions ordinarily used for protein hydrolysis is proved by the data of Table III. These data were obtained as previously described for the protein samples. The results show that small amounts of glucose, such as might be produced by hydrolysis of the polysaccharide in 100 mg. of many protein samples, give rise to about the same cysteine losses as the protein samples in Table I.

Table III also shows that the urea modification of Brand and Kassell (1) materially diminishes the amount of cysteine lost during acid digestion with glucose. The digests yielded by the sealed tube method were strongly

TABLE III

*Recovery of Cysteine Digested with Glucose in Acid Solution**

Initial glucose	Per cent recovery of cysteine	
	Sealed tube method	Urea method
mg.		
2	78.3	90.8
5	60.8	84.4
10	41.5	72.5

* The initial amount of cysteine was 5 mg. in each experiment.

colored and contained appreciable residues of insoluble humin. The urea digests were much paler and were entirely free from sediment.

In view of the effect of carbohydrates on cysteine, three of the proteins used in this study, ovalbumin, horse serum albumin, and lactoglobulin, were tested for their carbohydrate contents by the orcinol method of Sørensen and Haugaard (8). The results are given in Table IV. No attempt was made to determine the carbohydrate content of either wool or hair, owing to their insolubility. It has been shown, however, that wool, at least, contains a carbohydrate (10).

The values in Table IV do not necessarily indicate the total amounts of carbohydrate present, since glucosamine, which Neuberger (11) found in the polysaccharide of ovalbumin, does not respond to the orcinol test. The carbohydrate of horse serum albumin also appears to contain glucosamine (12). Sørensen (13) has shown that lactoglobulin contains not more than about 0.1 per cent glucosamine, and it is probably safe to assume that lactoglobulin is carbohydrate-free.

It is apparent from a comparison of Tables I, III, and IV that reaction between cysteine and carbohydrate can account for a large part of the loss of cysteine observed for those proteins which contain carbohydrate. The evidence indicates, however, that carbohydrate is not alone responsible, since we should then expect to observe a very marked improvement in recovery by the urea method, compared with the sealed tube method, by analogy with the results of Table III. The improvements in recovery revealed in Table I are much smaller than we should expect if the loss is all due to carbohydrate.

The best evidence that some protein component besides carbohydrate is involved is afforded by the results on lactoglobulin. Added cysteine is far from completely recoverable, although it is true that lactoglobulin gives the best recoveries of all the proteins investigated.

TABLE IV

Carbohydrate Content of Ovalbumin, Horse Serum Albumin, and Lactoglobulin; Orcinol Method

Protein	Per cent carbohydrate*
Ovalbumin.	2.32
Horse serum albumin.	1.27
Lactoglobulin.	0.02

* These values are given in terms of a mixture of equal parts of glucose and mannose, according to Kekwick (9). They are on a moisture-free basis.

Brand and coworkers (14) have quantitatively specified the composition of lactoglobulin in terms of known amino acid residues. To test the possibility that cysteine reacts with other amino acids during the digestion process, a "synthetic lactoglobulin" was prepared by mixing all the amino acids except cysteine in the proportions given by Brand. To 116 mg. of this mixture, equivalent to 100 mg. of protein, were added 5 mg. of cysteine and 2 ml. of 6 N HCl. Digestion was carried out by the sealed tube method. Recovery of cysteine was 95.7 per cent. Thus, the loss of cysteine, although probably significant, is small, and one must conclude that there is little reaction between cysteine and the other amino acids in lactoglobulin.

The next hypothesis tested was that the cysteine reacts with some peptide, although it is incapable of reacting with the free amino acids. If this were the case, cysteine added to an already hydrolyzed sample of lactoglobulin should be recoverable to the same degree as the cysteine added to the synthetic amino acid mixture. 100 mg. of lactoglobulin were therefore hydrolyzed by the sealed tube technique. To the hydrolysate were added 5 mg. of cysteine, the air was displaced by carbon dioxide, and the tube was

resealed while being evacuated, and heated for another 16 hours at 115°. The recovery of cysteine was 81.2 per cent, in excellent agreement with the value of 81.6 per cent obtained when the cysteine was added to the intact protein (Experiment 15, Table I). It is evident that the loss of cysteine is not attributable to reaction with a peptide. This experiment also proves that the substance responsible for the loss is not destroyed by the digestion process.

Another possibility is that there is some impurity in the lactoglobulin sample which is not a carbohydrate but is capable of reacting with cysteine. A test of this hypothesis was made by performing a recovery experiment by the sealed tube technique on a sample of lactoglobulin which had been re-crystallized four times.[2] A recovery of 84.8 per cent was obtained. This, although significantly higher than the 81.6 per cent recovery found for the once recrystallized material, still indicates a considerable loss of cysteine.

TABLE V

Recovery of Cysteine Added to Protein Hydrolysates

Protein*	Atmosphere during protein hydrolysis	Temperature during digestion with cysteine	Per cent recovery of cysteine
Horse serum albumin	Carbon dioxide	Boiling point	75.0
" " "	Air	" "	75.7
Wool	"	Room temperature	76.9
"	"	Boiling point	74.4

* The weight of each sample was 100 mg. 5 mg. of cysteine were added to each.

Thus, if some unidentified impurity which reacts with the cysteine is responsible, it is very difficult to separate this material from the protein by repeated crystallization. The possibility that it is not an impurity, but some as yet unrecognized part of the molecule, is not to be ignored.

As in the case of lactoglobulin, tests were made on horse serum albumin and wool to determine whether the recovery of cysteine added after hydrolysis of the protein is different from the recovery of cysteine added to the intact protein. These determinations, reported in Table V, were made by the urea method. To determine whether the hypothetical substance which reacts with the cysteine is stable to air oxidation, an experiment was performed on horse serum albumin in which the hydrolysis of the protein which preceded the addition of cysteine was carried out in one case in air and, in the other, under carbon dioxide. The subsequent digestion with the cysteine was performed under carbon dioxide in every case. To determine whether the reaction which leads to the loss of cysteine takes place at room

[2] We should like to thank Dr. R. C. Warner of this Laboratory for this sample.

temperature as well as at the boiling point, one experiment was carried out on wool, in which the mixture of wool hydrolysate and added cysteine was allowed to stand for 24 hours at room temperature. In the other experiments, the mixture of hydrolysate and added cysteine was refluxed for 6 hours.

Comparison of Table V with Table I confirms the conclusion previously reached for lactoglobulin; namely, that it makes little difference whether the cysteine is added to the protein before or after the latter is hydrolyzed. The interfering substance resists the action of acids, and is stable toward air oxidation. The reaction with cysteine takes place nearly to the same extent at room temperature as at the boiling point of the digestion mixture. Dialysis of a hydrolyzed lactoglobulin sample showed that the interfering substance passes through a cellophane dialyzing membrane.

DISCUSSION

The chief conclusion to which this study leads is that the determination of cysteine in a protein by a method involving acid hydrolysis is subject to serious error, an error, moreover, which it is difficult to determine quantitatively. In many analytical procedures which involve small losses, it is a common practice to add small amounts of the constituent sought, submit the mixture to the analytical procedure, calculate the per cent recovery of the added substance, and use this recovery factor to correct the value obtained for the desired constituent in the absence of any additions. This is especially true of samples of biological origin. Such a procedure is feasible, however, only when the correction is small (15 per cent is probably about the upper limit) and reasonably constant. In this case, however, the corrections are so large and so dependent on the amount of the desired constituent that no reliable correction seems possible.

It has been shown that only part of the loss can be attributed to reaction with carbohydrates. Part seems to be due to an unknown non-carbohydrate compound. The possibility that the unknown compound is an aldehyde was considered, but the fuchsin test applied to hydrolyzed lactoglobulin was negative. It is not claimed that the incomplete recovery shown by carbohydrate-free lactoglobulin proves the existence of an unidentified component of the molecule. It is conceivable that lactoglobulin actually is composed of nothing but known amino acids but that, during hydrolysis, small amounts of compounds other than amino acids are produced, compounds the formation of which depends on the spatial arrangement of the amino acids in the structure and which are, therefore, not produced by the digestion of a mixture of free amino acids.

If cysteine is lost during protein hydrolysis, as Tables I and V indicate, it

is necessary to explain the fact that for a number of proteins the determined amounts of cysteine, cystine, and methionine account satisfactorily for the total protein sulfur (1, 15–19). Most of these proteins, however, either contain no cysteine, or the contribution of cysteine to the total sulfur is so small that an error of 20 per cent in the cysteine estimation would not significantly affect the total sulfur. In the above references, of seventeen protein samples for which total sulfur is compared with the sum of cysteine, cystine, and methionine sulfur, there are only four for which correction of the cysteine content for an assumed 20 per cent loss disturbs the sulfur balance to any considerable degree. These are lactoglobulin (18), chymotrypsinogen (1), egg albumin (19), and myosin (19).

It may be that the lost cysteine is converted wholly or in part to cystine. This would make it possible for the total sulfur to be correct in spite of the fact that the cysteine is too low, since the sum of cysteine and cystine would be constant. Several experiments were performed by the authors to determine whether, in some of the experiments shown in Table I, the missing cysteine could be accounted for as cystine. The results were inconclusive, owing to the difficulty of determining a small change in cystine content of a solution containing relatively large amounts of both cystine and cysteine.

Regardless of the results of sulfur balance determinations, it is obvious that values for cysteine obtained by acid hydrolysis methods must be looked on with grave suspicion unless the method can be so modified as to give complete recoveries of added cysteine.

In view of the difficulties involved in acid hydrolysis, it may be best to seek some method for determining sulfhydryl which utilizes the intact protein. A beginning toward such a method has been made by Todrick and Walker (20), Kuhn and Desnuelle (21), and Mirsky and Anson (22).

SUMMARY

1. Cysteine added to proteins cannot be completely recovered after the protein is hydrolyzed by acid digestion, making doubtful cysteine determinations by this method.

2. Hydrolysis in the presence of urea prevents insoluble humin formation but only slightly improves the recovery of added cysteine.

3. Approximately the same loss of cysteine occurs if it is digested with a prehydrolyzed rather than an intact protein.

4. The presence of carbohydrate in some of the protein samples accounts partially but not completely for the loss of cysteine.

5. Lactoglobulin, a carbohydrate-free protein, reacts with cysteine during acid digestion, but a supposedly equivalent mixture of free amino acids does not.

BIBLIOGRAPHY

1. Brand, E., and Kassell, B., *J. Gen. Physiol.*, **25**, 167 (1941).
2. Lugg, J. W. H., *Biochem. J.*, **27**, 1022 (1933).
3. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938).
4. Adair, G. S., and Robinson, M. E., *Biochem. J.*, **24**, 993 (1930).
5. Sørensen, S. P. L., and Høyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, **12**, 12 (1917).
6. Sørensen, M., and Sørensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **23**, 55 (1939).
7. Schubert, M. P., *J. Biol. Chem.*, **130**, 601 (1939).
8. Sørensen, M., and Haugaard, G., *Compt.-rend. trav. Lab. Carlsberg*, **19**, 1 (1933).
9. Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938).
10. von Allwörden, K., *Z. angew. Chem.*, **29**, 77 (1916).
11. Neuberger, A., *Biochem. J.*, **32**, 1435 (1938).
12. Rimington, C., *Biochem. J.*, **23**, 430 (1929).
13. Sørensen, M., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **22**, 487 (1938).
14. Brand, E., Saidel, L. J., Goldwater, W. H., Kassell, B., and Ryan, F. J., *J. Am. Chem. Soc.*, **67**, 1524 (1945).
15. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 435 (1938).
16. Brand, E., Kassell, B., and Heidelberger, M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. xi (1939).
17. Brand, E., Cahill, G. F., and Kassell, B., *J. Biol. Chem.*, **125**, 415 (1938).
18. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 365 (1942).
19. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **151**, 635 (1943).
20. Todrick, A., and Walker, E., *Biochem. J.*, **31**, 292 (1937).
21. Kuhn, R., and Desnuelle, P., *Z. physiol. Chem.*, **251**, 14 (1938).
22. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **18**, 307 (1934).

RELATION OF AMINO ACID IMBALANCE TO NIACIN-TRYPTO-
PHANE DEFICIENCY IN GROWING RATS*

By W. A. KREHL, L. M. HENDERSON, J. DE LA HUERGA,
AND C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of
Wisconsin, Madison*)

The growth inhibition which results from the inclusion of corn in a low
protein, niacin-deficient ration (1) and the apparent dual rôle of niacin and
tryptophane in counteracting this growth depression have resulted in con-
siderable study on the mechanism of these relationships. It has been
indicated that intestinal microorganisms may play a significant part in this
deficiency syndrome, since the deleterious effect of corn can be greatly
modified by the use of carbohydrates previously shown to favor intestinal
synthesis. The suggestion has also been made that corn might contain a
substance structurally analogous to niacin which would compete with
this vitamin (2). The observation that tryptophane administration results
in the increased excretion of niacin and niacin derivatives (3) indicates that
the similar growth-promoting activity of niacin and tryptophane may be
due to the fact that tryptophane functions as a metabolic precursor of
niacin. The fact that niacin synthesis is greater on a synthetic ration
than when corn is added, despite the fact that the former ration contains
slightly less tryptophane than the latter (1), does not lend credence to the
explanation that tryptophane acts as a precursor of niacin under these
conditions. It has been demonstrated, however, that niacin may improve
the animal's production or utilization of tryptophane (4). If the synthesis

of niacin is impaired or if niacin is supplied in inadequate amounts, the
dietary requirement for tryptophane is increased.

Considerable evidence is at hand to show that the chief protein of corn
(*i.e.*, zein), other proteins such as gelatin, and acid-hydrolyzed proteins,
free of tryptophane, create a syndrome exactly analogous to that produced
by corn (5). These results indicate that the growth depressions caused by
corn and the non-corn-containing rations which contain tryptophane-free
proteins have a common basis; namely, an imbalance of amino acids.

Attempts have been made in the present study to extend our information
concerning the deleterious action of corn grits and the counteraction by
various materials and to elucidate the nature of the amino acid imbalance
conducive to the growth failure with rations which contain added protein
or protein hydrolysates free of tryptophane.

<div align="center">EXPERIMENTAL</div>

The composition of the diets used is given in Table I. The vitamins
were added in the following amounts per 100 gm. of ration: thiamine 0.2
mg., riboflavin 0.3 mg., pyridoxine 0.25 mg., calcium pantothenate 2.0 mg.,
choline 100 mg., 2 methyl-1,4-naphthoquinone 0.1 mg., inositol 10 mg.,
and biotin 0.01 mg. Vitamins A and D were supplied as halibut liver oil,
2 drops every week, together with α-tocopherol, 1 mg. per rat per week.
Unless otherwise indicated, the basal rations contained practically no nico-
tinic acid. Weanling male rats of the Sprague-Dawley strain were used in
all cases and each group contained three rats.

In an effort to determine whether an insoluble sulfonamide would inhibit
the effect of niacin in corn grits rations, sulfasuxidine was added with and
without niacin. Synthetic *Lactobacillus casei* factor was also added at
a level of 25 γ per 100 gm. of ration, since a deficiency of this factor is
known to occur in the rat in the presence of sulfonamides (6). The
results for Groups 1 and 2 in Table II show that sulfasuxidine does not
counteract the beneficial action of niacin. In a similar experiment,
streptothricin was added to the corn grits ration at a level of 25,000 units
per 100 gm. of ration and it likewise failed to impair the beneficial action of
niacin. If it is assumed that niacin favors the intestinal synthesis of
tryptophane it appears that the above drugs did not impair the activity of
organisms responsible for this synthesis. This is not surprising in view of
the fact that these drugs have been shown to alter the synthesis of some but
not all factors.

In an experiment to test the value of natural foodstuffs in counteracting
the action of corn grits two rations were devised containing peanuts and
brewers' yeast respectively. These rations were prepared by reducing the
casein content so that the tryptophane level was approximately the same as

that in the usual corn grits ration. In this manner the effectiveness of these supplements could be tested, based on their niacin content, which for the amounts used was about 1.5 mg. per cent for both the peanut and brewers' yeast rations. The results shown in Table II, Groups 3 and 4, indicate the beneficial value of these supplements in the corn grits diet.

In a report on the nutritive value of the Mexican tortilla (7), it was indicated that this food, made from corn, provided a major portion of the Mexican dietary. Pellagra, while not unknown in Mexico, does not appear extensively as a result of this diet. Tortillas were prepared,

TABLE I

Composition of Basal Ration

Ration constituent	Ration No.								
	1	2	3	4	5	6	7	8	9
Sucrose	78	84		38	43		82	82	82
Dextrin			84			73			
Casein*	15	9	9	7	8	9	9	9	9
Gelatin						6			
Zein						3			
Fibrin hydrolysate						2	2		
Casein "								2	
Egg albumin hydrolysate									2
Peanuts (ground)				10					
Brewers' yeast					4				
Corn grits				40	40				
" oil	3	3	3	2	2	3	3	3	3
Salts IV†	4	4	4	3	3	4	4	4	4
l(−)-Cystine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

* Crude casein extracted three times with boiling 95 per cent ethanol.
† Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109,** 657 (1935).

therefore, according to the method described by Cravioto (7), dried, and added to the basal ration in the usual manner. It can be seen that dried tortillas inhibited growth just as severely as corn grits (Table II, Group 6) and that niacin completely counteracted this inhibition. Since the most significant difference between the Mexican tortilla and whole yellow corn is the much higher calcium content of the tortilla, the yellow corn ration was supplemented with 0.5 per cent $CaCO_3$. Marked growth inhibition was again evident (Table II, Group 5), which was corrected with niacin.

In a previous report (1) it was shown that, of the various cereals tested, only corn inhibited growth under the conditions used. Additional experiments with cow peas, which are used extensively in the South, and with

TABLE II

Growth Results on Various Diets

Group No.	Basal ration (Table I)*	Type of ration† and supplement to basal ration	Gain per wk. for 4 wks., 3 rats per group‡
			gm.
1	1	Corn grits 40%	9
2	1	" " 40 % + 2% sulfasuxidine	4 (+N, 29)
3	4	(" " 40%, peanut)	31
4	5	(" " 40%, brewers' yeast)	29
5	1	Yellow corn, 40% + CaCO₃, 0.5%	7 (+N, 30)
6	1	Dried tortillas 40%	9 (+N, 27)
7	1	Cow peas 40%	28
8	1	Corn bran 40%	30
9	1	" grits 40% + 5% corn germ	28
10	1	" " 40% + 10% " "	25
11	1	" " 40% + 3% dried pig stomach	27
12	1	" " 40% + 3-CN pyridine 1.5 mg. %	7 (+N, 32)
13	1	" " 40% + indole 125 mg. %	4 (+T, 33)
14	1	" " 40% + anthranilic acid 100 mg. %	7 (+T, 33)
15	1	" " 40% + indole-3-acetic acid 100 mg. %	4 (+T, 31)
16	2	(Sucrose)	15 (+N, 18)
17	3	(Dextrin)	27
18	2	(Sucrose) + pyridine-3-sulfonic acid 0.2%	9 (+N, 15)
19	2	" + 3-acetylpyridine · HCl 0.2%	17
20	6	(Dextrin-casein 9%, gelatin 6%, zein 3%, fibrin hydrolysate 2%)	11 (+N, 32) (+T, 29)
21	3	(Dextrin) + glycine 1%	25
22	3	" + l(−)-leucine 1%	24
23	3	" + l(+)-glutamic acid 1%	23
24	3	" + glycine, l(−)-leucine, and l(+)-glutamic acid 1% each	27 (+N, 27) (+T, 24)
25	2	(Sucrose) + glycine 1.5%, l(−)-leucine 1%	8 (+N, 18)
26	2	" + l(+)-glutamic acid 1.5%, l(−)-leucine 1%	12 (+N, 21)
27	2	(Sucrose) + glycine 1.5%, l(−)-leucine 1.0%, l(−) tyrosine 0.25%, dl-phenylalanine 0.5%, dl-valine 0.25%	5 (+N, 13)
28	2	(Sucrose) + l(+)-glutamic acid 1.5%, l(−)-tyrosine 0.25%, dl-phenylalanine 0.5%, dl-valine 0.25%	11 (+N, 16)

* Supplements of corn grits, yellow corn, dried tortillas, cow peas, and corn bran were added at the expense of the entire basal ration indicated. Other supplements were added at the expense of sucrose, except 3-CN pyridine, indole, anthranilic acid, indole-3-acetic acid, pyridine-3-sulfonic acid, and 3-acetylpyridine hydrochloride which were added to the entire basal ration.

† Parentheses indicate the type of ration.

‡ The letters N and T in parentheses indicate oral supplementation with 0.25 mg. of niacin and 10 mg. of dl-tryptophane per rat per day respectively. The accompanying figures are the average weekly gains during the 4th and 5th weeks when supplements were given. The figures outside of the parentheses represent average weekly gains during the first 3 weeks. A control rat from each group was continued on the deficient or niacin-low ration.

corn bran show that these materials fed at a level comparable to corn grits produce no growth inhibition (Table II, Groups 7 and 8).

The excellent nutritive quality of corn germ is evident by the fact that when it was included in the corn grits ration at a level as low as 5 per cent (at the expense of sucrose) good growth was obtained. In considering the somewhat surprising effectiveness of such low levels of corn germ it is worth while to note the narrow range in which the level of tryptophane is active in counteracting growth inhibition. For example when wheat gluten and gelatin were used as the protein source (1) and the tryptophane content was 120 mg. per cent, poor growth resulted, but the same ration allowed good growth when the tryptophane level was increased to only 145 mg. per cent. The effectiveness of corn germ may be due in part to the fact that it contributes several other of the amino acids which may be present in the corn grits ration in borderline amounts, although its protective action is perhaps better explained by its higher niacin (*i.e.* about 40 to 45 γ per gm.) and tryptophane content (see Table II, Groups 9 and 10). Dried pig stomach, a good source of both niacin and tryptophane, gave excellent protection against corn grits (see Table II, Group 11).

Certain compounds related to niacin or tryptophane were tested for their ability to counteract the growth deficit caused by corn grits. It has been shown that 3-CN pyridine is unable to cure canine blacktongue (8) and it is clear that it has no niacin activity in the rat, although when niacin was given after 3 weeks to rats receiving 3-CN pyridine a marked growth response was observed (Table II, Group 12). Indole and indole-3-acetic acid were tested for tryptophane activity in counteracting the deleterious effect of corn and found to be completely inactive. No competitive activity of these compounds against tryptophane was observed since added *dl*-tryptophane, at a level of 10 mg. per rat per day, resulted in excellent growth. Anthranilic acid was tested in a similar manner because it is known to be active for certain bacteria (9), but it too was inactive, although it did not prevent the beneficial action of tryptophane. (The results are given in Table II, Groups 13 to 15).

Woolley reported the antiniacin activity of certain compounds related to niacin and showed that 3-acetylpyridine given orally in single test doses created a niacin deficiency in the mouse (10) which was counteracted by large doses of niacin. In a similar experiment with pyridine-3-sulfonic acid, no niacin inhibition was observed even at very high levels of this compound (11).

In the present study 3-acetylpyridine as the hydrochloride was incorporated in the ration at a level of 0.2 per cent with no untoward effects (compare Group 19 with Group 16, Table II). Whether this difference is due to species variation or the somewhat different manner in which the

compound was given is uncertain. When pyridine-3-sulfonic acid was included in the same ration at a level of 0.2 per cent (9 per cent casein, sucrose basal) some growth retardation was observed which was counteracted by niacin (Table II, Group 18). In earlier experiments this compound had been fed to rats at levels up to 3 per cent with no harmful effects. However, at that time we were using a ration containing 20 per cent casein. This difference in behavior of the sulfonamide may be largely due to the difference in the protein level of the rations. Woolley has shown, for example, that the antiniacin action of 3-acetylpyridine can be counteracted with tryptophane (2).

In a continuation of the previous study with non-corn, low protein rations (5) experiments were conducted to study the growth inhibition caused by the addition of proteins or protein hydrolysates free of tryptophane. In verification of previous results (5), dextrin produced better growth than sucrose when the basal ration contained 9 per cent casein (Table II, Groups 16 and 17). However, the presence of dextrin did not protect the rat from the growth inhibition which resulted when tryptophane-free proteins or protein hydrolysates, such as a combination of 6 per cent gelatin, 3 per cent zein, and 2 per cent acid-hydrolyzed fibrin, were added (Table II, Group 20), despite the fact that the nitrogen content was increased to a level corresponding to about 20 per cent protein.

That the growth inhibition induced by acid-hydrolyzed proteins such as fibrin, egg albumin, and casein at levels of 2 per cent in a 9 per cent casein ration can be immediately counteracted by niacin, even after 3 weeks on the inhibitory regimen, is evident in Fig. 1, growth Curves B, C, and D. The control rats on these rations which received no niacin grew very little. It is also clear that when niacin is added the slopes of the growth curves are the same as those obtained with rations containing no supplement of acid-hydrolyzed protein (Fig. 1, Group A). The addition of niacin to the latter ration stimulates growth only slightly.

Inasmuch as one or more of the amino acids, glycine, leucine, and glutamic acid, are present in rather large amounts in the tryptophane-low proteins, zein and gelatin, and in the tryptophane-free acid-hydrolyzed proteins, fibrin and casein, these amino acids were added separately and collectively at a level of 1 per cent each to the 9 per cent casein-dextrin ration. No growth inhibition could be observed from these amino acids under the conditions employed (Table II, Groups 21 to 24). When sucrose was used as the carbohydrate, however, and glycine and $l(-)$-leucine added together at a level of 1.5 and 1 per cent respectively, a growth inhibition was observed which was corrected by the addition of niacin at a level of 250 γ per rat per day. A combination of glutamic acid and leucine at levels of 1.5 and 1 per cent respectively produced slightly less growth than that

obtained with the 9 per cent casein-sucrose basal, but the addition of niacin resulted in marked growth stimulation. The addition of 0.25 per cent $l(-)$-tyrosine, 0.5 per cent dl-phenylalanine, and 0.25 per cent dl-valine to the above combinations of glycine and $l(-)$-leucine seemed to depress growth still more and the addition of niacin was much less effective in
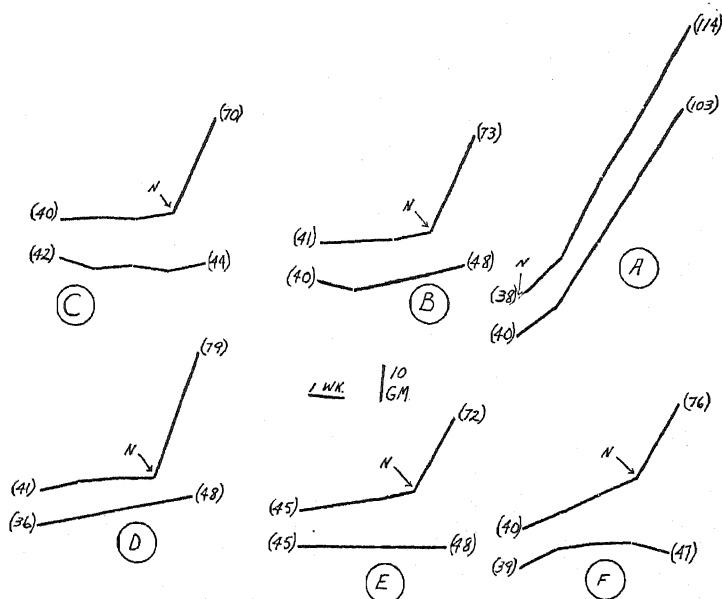
Fig. 1. Growth responses in rats on various diets when supplemented with niacin (N) at the indicated point in the growth curve.   Group A, 9 per cent casein (sucrose) basal with and without niacin; Group B, 9 per cent casein (sucrose) basal plus 2 per cent acid-hydrolyzed fibrin; Group C, 9 per cent casein (sucrose) basal plus 2 per cent acid-hydrolyzed egg albumin; Group D, 9 per cent casein (sucrose) basal plus 2 per cent acid-hydrolyzed casein; Group E, 9 per cent casein (sucrose) basal plus 6 per cent gelatin; and Group F, 9 per cent casein (sucrose) basal plus 2 per cent glycine.

counteracting this inhibition (for the results consult Table II, Groups 25 to 28).

The growth inhibition caused by 6 per cent gelatin and the counteraction by niacin are shown in Fig. 1, Group E.   That glycine is intimately concerned in the amino acid imbalance involved in these studies is indicated by the fact that the addition of 2 per cent glycine to the sucrose-casein ration tends to retard growth (Fig. 1, Group F) and niacin counteracts this retardation.   It should be noted, however, that glycine caused very little, if any, growth inhibition when dextrin was used as the carbohydrate.

One of the observations made upon autopsy of rats which received corn

without added niacin was the fact that the livers appeared fatty and friable. When the fat content of these livers was determined, it was found that the animals which received corn had, as a rule, more liver fat than the animals receiving added niacin. On analysis of the livers from the deficient animals for niacin (12) it was found that they contained less of this vitamin than the control animals. That the level of choline has a bearing on the problem of liver fat is evident from the fact (Table III)

TABLE III

Niacin and Fat Content of Rat Livers on Various Diets

Basal ration (Table I)	Supplement to basal ration*	Liver niacin				Liver fat	
		−N†	+N†	−T‡	+T‡	−N†	+N†
		γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent	per cent
1	(Sucrose basal) (choline 100 mg. %)	160	165			3.0	2.8
1	+ 40% corn grits	110	164			8.4	3.2
1	+ 40% yellow corn (choline 60 mg. % in ration)	116	146			19	3.3
1	+ 40% corn grits + indole-3-acetic acid			80	136		
1	+ 40% " " + 3-CN pyridine	84	108				
1	+ 40% " " (choline 10 mg. % in ration)	84	120			25	20
1	+ 40% corn grits (choline 30 mg. % in ration)	86	86			22	18
1	+ 40% corn grits (choline 60 mg. % in ration)	110	126			17	9.7
2	(Sucrose) + 3% gelatin	98	105		159		
2	" + 3% zein	77	108				
2	" + 2% acid-hydrolyzed fibrin	97	108		141		

* The parentheses indicate the type of ration.

† −N and +N refer to no niacin in the ration and added niacin at a level of 1.5 mg. per cent.

‡ −T and +T refer to no added tryptophane or added tryptophane at a level of 50 mg. per cent.

that, as the choline content of the ration is decreased, the liver fat markedly increased, even though the animals still received a favorable growth response when niacin was added. However, the niacin content of the liver remained low as long as liver fat was high.

It was interesting to note (Table III) that the niacin content of the livers from deficient animals which received the growth-inhibiting proteins, gelatin and zein, or acid-hydrolyzed fibrin was low and was not greatly increased until the addition of niacin. When tryptophane was added, however, the content of niacin in the liver markedly increased. This would indicate that tryptophane may play a direct rôle in niacin synthesis (3).

DISCUSSION

The evidence so far obtained indicates that the action of corn in niacin-low synthetic rations is a function of the character of the predominant protein present, zein, and that this, in turn, is due to an amino acid imbalance in this protein. It seems that this imbalance is made apparent through growth depression under any set of circumstances which provides too little tryptophane and niacin. That an imbalance of amino acids results in an increased tryptophane requirement, and therefore poor growth, is evident by the fact that rats will grow reasonably well on rations which contain the same amount of tryptophane but which also contain a well balanced protein. This increased requirement could, however, be related to a greatly reduced supply of niacin, through inadequate synthesis, which results in decreased tryptophane utilization or production.

It is clear that the kind of carbohydrate used in the ration influences the results obtained. The very much better growth obtained with dextrin as compared with sucrose even on the basal ration probably indicates one of three things: (1) dextrin is stimulating the synthesis of additional factors needed by the rat; (2) dextrin not only provides desirable conditions in the intestinal tract for bacterial activity but, because of its slow hydrolysis and absorption, favors a more gradual absorption and better utilization of other accompanying nutrients; and (3) dextrin may carry some unidentified factor *per se*. A combination of the first two postulations seems more reasonable.

Albanese and Irby (13) early reported the inferiority of essential amino acids for growth as compared with protein hydrolysates and implicated the unnatural forms of certain amino acids for this growth failure. Kinsey and Grant (14) indicated that high levels of glycine were toxic and Hier *et al.* (15) also showed that gelatin and the amino acids, glycine, phenylalanine, and proline, resulted in growth inhibition. The levels used in these instances were undoubtedly toxic since adequate amounts of niacin were included in their rations.

Although glycine of the amino acids tested seems to inhibit growth the most, this cannot be the only amino acid involved, since acid-hydrolyzed casein and zein, both of which inhibit growth, contain little or no glycine. This does not preclude the possibility that more than one amino acid may be involved in the imbalance of amino acids in such circumstances.

SUMMARY

Natural materials, such as peanuts, brewers' yeast, corn germ, corn bran, and dried pig stomach, used as supplements to the corn grits ration under controlled conditions, adequately prevent growth inhibition.

Compounds such as 3-CN pyridine, indole, indole-3-acetic acid, and anthranilic acid did not replace either niacin or tryptophane in counter-

acting the deleterious action of corn grits, but neither did they compete with niacin or tryptophane to reduce their beneficial action.

Some inhibition of growth, which was corrected by niacin, was observed by pyridine-3-sulfonic acid in rations containing a low level of protein but not at a high protein level.

An amino acid imbalance in zein and other tryptophane-low proteins creates a growth depression on niacin-low diets, and, of the specific amino acids so far tested, glycine seems to be one of the amino acids most responsible. This effect is dependent to some extent on the type of carbohydrate used, since glycine exhibits no inhibition when dextrin is used in place of sucrose. In all cases, dextrin rations produced markedly superior growth to sucrose rations.

BIBLIOGRAPHY

1. Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A., *J. Nutr.*, **31**, 85 (1946).
2. Woolley, D. W., *J. Biol. Chem.*, **162**, 179 (1946).
3. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **163**, 343 (1946).
4. Krehl, W. A., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **164**, 551 (1946).
5. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 403 (1946).
6. Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 713 (1942).
7. Cravioto, R. O., Anderson, R. K., Lockhart, E. E., Miranda, F. P., and Harris, R. S., *Science*, **102**, 91 (1945).
8. Woolley, D. W., Strong, F. M., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, **124**, 715 (1938).
9. Snell, E. E., *Arch. Biochem.*, **2**, 389 (1943).
10. Woolley, D. W., *J. Biol. Chem.*, **157**, 455 (1945).
11. Woolley, D. W., and White, A. G. C., *Proc. Soc. Exp. Biol. and Med.*, **52**, 106 (1943).
12. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 471 (1943).
13. Albanese, A. A., and Irby, V., *Science*, **98**, 286 (1943).
14. Kinsey, V. E., and Grant, W. M., *Science*, **99**, 303 (1944).
15. Hier, S. W., Graham, C. E., and Klein, D., *Proc. Soc. Exp. Biol. and Med.*, **56**, 187 (1944).

AN IMPROVED SYNTHESIS OF β-2-THIENYLALANINE

By KARL DITTMER, WERNER HERZ, AND JOHN S. CHAMBERS

(From the Department of Chemistry, University of Colorado, Boulder)

The inhibition of microbial growth by 2-thienylalanine as an antagonist of phenylalanine was demonstrated by du Vigneaud and coworkers (1). Recently more detailed studies of this inhibition were reported (2, 3).

Further experimentation with this compound should be profitable if it were more readily available. The reported methods (1, 4) for the preparation of 2-thienylalanine are time-consuming. Since 2-chloromethylthiophene can be prepared easily from thiophene by the method of Blicke and Burckhalter (5), we investigated the possibility of applying the phthalimidomalonic ester (6) and the acetamidomalonic ester (7, 8) syntheses to the preparation of 2-thienylalanine. By these methods relatively large amounts of this substance were conveniently obtained, in good yields.

EXPERIMENTAL

Diethyl Thenylphthalimidomalonate—15.0 gm. of sodiophthalimidomalonic ester (9) dissolved in 40 cc. of dry toluene, and 5.9 gm. of 2-chloromethylthiophene (thenyl chloride) prepared by the method of Blicke and Burckhalter (5), were refluxed for 6 hours. The sodium chloride was filtered off and the filtrate was concentrated *in vacuo* to remove all toluene. After a little alcohol had been added to the residue the product crystallized. The yield was 16.5 gm. of crude material (93 per cent of theory).

A small amount was purified for analysis by recrystallizing from an alcohol-water mixture. A second recrystallization from acetone and water yielded shiny white platelets, m.p. 106.5–107° (uncorrected).

$C_{20}H_{19}O_6NS$ (401.4)   Calculated, N 3.49; found, N 3.49

Diethyl Thenylacetamidomalonate—4.35 gm. of sodium, purified by boiling in toluene, were added to 200 cc. of absolute ethanol. After the sodium had dissolved completely, 41 gm. of acetamidomalonic ester (7) were added and then, in one portion, 25 gm. of 2-chloromethylthiophene. The mixture was refluxed for 4 hours; on cooling some of the product separated. It was collected on the filter and washed with cold water to remove the salt. The filtrate was concentrated to about 50 cc. and water was added until the crystals separated. The product was collected,

541

recrystallized from alcohol and water, and combined with the first fraction. Yield, 42 gm., m.p. 112–115°. The mother liquors yielded an additional 9.7 gm., m.p. 108–114°. The total yield, therefore, was 51.7 gm. (87.7 per cent of theory).

For analysis a small sample was recrystallized once more from boiling water. M.p. 116° (uncorrected).

$$C_{14}H_{19}O_5NS \ (313.3) \quad \text{Calculated, N 4.47; found, N 4.57}$$

β-2-Thienylalanine. From Diethyl Thenylphthalimidomalonate—A mixture consisting of 16.5 gm. of the crude diethyl thenylphthalimidomalonate and 20.5 gm. of barium hydroxide in 60 cc. of water was refluxed for 9 hours. The solution was acidified with dilute sulfuric acid to pH 2 with evolution of gas, heated on the steam bath until no more gas was evolved, and allowed to stand overnight. The barium sulfate was filtered off and thoroughly washed with boiling water. The combined filtrate and washings were freed of barium and sulfate ions, and concentrated to 10 cc. *in vacuo.* The residue was diluted with 50 cc. of alcohol; when crystals started to separate, 10 cc. of ether were added. 3.1 gm. of thienylalanine melting at 273° were obtained. The mother liquors were concentrated to dryness, again dissolved in a small amount of water, and diluted with alcohol and ether as before. An additional 0.8 gm. of product was obtained. The total yield was 3.9 gm., representing 51 per cent of theory, based on the 2-chloromethylthiophene used.

From Diethyl Thenylacetamidomalonate—41 gm. of the crude diethyl thenylacetamidomalonate were refluxed with 200 cc. of 48 per cent hydrobromic acid for 7 hours. Most of the HBr was removed *in vacuo;* the remainder was neutralized to pH 6 with ammonium hydroxide. The entire solution was concentrated to dryness and the ammonium bromide was washed out of the residue with 40 cc. of ice water, followed by washing with alcohol and ether. The crude thienylalanine free of salt weighed 17.3 gm., representing a 78 per cent yield, based on diethyl thenylacetamidomalonate, which is an over-all yield of 67.5 per cent, based on 2-chloromethylthiophene.

For further purification, 18.3 gm. of the crude material were dissolved in 50 cc. of 10 per cent hydrobromic acid. The solution was decolorized with charcoal, filtered, and the acid neutralized by the calculated amount of ammonium hydroxide. The neutral solution was diluted with 5 volumes of ethanol and 1 volume of ether and cooled overnight. 13.1 gm. of pure product were obtained. An additional 3.2 gm. of less pure material were recovered from the filtrate.

$$C_7H_9O_2NS \ (171.2) \quad \text{Calculated, N 8.18; found, N 8.25}$$

The authors wish to thank Dr. John S. Meek of this laboratory for a generous quantity of acetamidomalonic ester.

SUMMARY

β-2-Thienylalanine has been prepared from 2-chloromethylthiophene by the use of phthalimidomalonic ester and acetamidomalonic ester in 51 and 67.5 per cent over-all yields respectively.

BIBLIOGRAPHY

1. du Vigneaud, V., McKennis, H., Jr., Simmonds, S., Dittmer, K., and Brown, G. B., *J. Biol. Chem.*, **159**, 385 (1945).
2. Dittmer, K., Ellis, G., McKennis, H., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **164**, 761 (1946).
3. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **164**, 53 (1946).
4. Barger, G., and Easson, A. P. T., *J. Chem. Soc.*, 2100 (1938).
5. Blicke, F. F., and Burckhalter, J. H., *J. Am. Chem. Soc.*, **64**, 477 (1942).
6. Sörensen, S. P. L., *Z. physiol. Chem.*, **44**, 448 (1905).
7. Snyder, H. R., and Smith, C. W., *J. Am. Chem. Soc.*, **66**, 350 (1944).
8. Albertson, N. F., Archer, S., and Suter, C. M., *J. Am. Chem. Soc.*, **66**, 500 (1944); **67**, 36 (1945). Albertson, N. F., and Archer, S., **67**, 308 (1945).
9. Barger, G., and Weichselbaum, T. E., in Organic syntheses, New York, coll. 2, 384 (1943).

FURTHER STUDIES OF MUTANT STRAINS OF NEUROSPORA REQUIRING ISOLEUCINE AND VALINE*

By DAVID BONNER†

(From the School of Biological Sciences, Stanford University, California)

Investigations of biochemical mutations in *Neurospora* support the thesis that there exists a 1:1 relation between chemical reaction and gene (2, 5, 10). A single gene mutation therefore might be expected to block a single reaction, possibly by conditioning the formation of a single enzyme. While a single enzyme may catalyze a number of reactions of a common type, general experience with mutants of *Neurospora* is that a single gene mutation results in the impairment or failure of but a single chemical reaction. An apparent exception to this general finding has been reported previously (7). Strain 16117, which differs by a single gene from the parent strain, was reported to have a growth requirement for the two amino acids, isoleucine and valine. To account for this double requirement, associated with a single gene change, it was suggested that strain 16117 was genetically blocked in a common step in the synthesis of these two closely related β-methylamino acids. Continued investigation of this, and other closely related strains, has given support to the general concept of 1:1 correspondence, although the particular mechanism suggested by Bonner *et al.* (7) appears now to be incorrect.

A general scheme of isoleucine and valine synthesis is presented in Fig. 1. The work described in this paper constitutes the evidence for this scheme. The scheme shows the synthesis of isoleucine in strain 16117 to be genetically blocked in the conversion of ketoisoleucine[1] to isoleucine, and the block in valine synthesis to be due to the inhibition of valine synthesis by accumulated ketoisoleucine. Thus the association of a single gene difference with a double growth factor requirement is accounted for by the inhibition of a reaction in one synthesis by an intermediate accumulated as a result of a genetic block in a second.

[1] The terms ketoisoleucine and ketovaline are used to refer to α-keto-β-methyl-*n*-valeric acid and α-ketoisovaleric acid respectively.

545

Methods

Quantitative estimation of growth response was made by culturing the strain 72 hours at 25° in 125 ml. Erlenmeyer flasks containing 20 ml. of medium. After incubation the mycelial pad was removed, dried at 100°, and weighed.

The basal medium used throughout is that described elsewhere (4, 9).

In general, additions to the basal medium were made prior to sterilization. That this procedure is justified is shown by the fact that no significant difference in activity was noted when keto acid solutions were sterilized by filtration and then added to sterile basal medium.

Ketoisoleucine and ketovaline were both prepared synthetically. α-Keto-isovaleric acid was prepared from diethyl dimethyloxalacetate as described by Rassow and Bauer (12). α-Keto-β-methyl-n-valeric acid was prepared
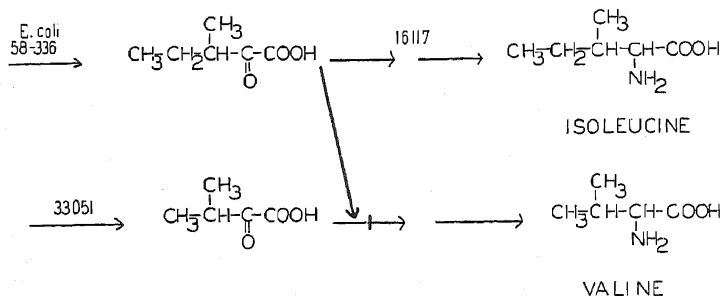
FIG. 1. A scheme of isoleucine and valine synthesis in *Neurospora*. Genetic blocks are indicated by strain numbers.

from diethyl methylethyloxalacetate according to the method of Mebus (11). The calcium salts of both compounds were prepared for growth tests.

Strains—Five strains in addition to those previously reported having the double requirement for isoleucine and valine have been obtained. All available biochemical data suggest that these strains are genetically identical. Heterocaryon tests (3) among these five strains were indeterminate. Two strains, Nos. 39709 and 46807, which heterocaryon tests indicated might differ from strain 16117, were crossed with normal strains and with strain 16117. Crosses of these strains with normal strains give segregation expected of a single gene difference. Crosses with strain 16117 indicate genetic identity of these three strains, though the possibility of closely linked non-allelic genes cannot be ruled out. Genetic and biochemical evidence, therefore, favors the view that these strains are recurrences of the same mutation present in strain 16117.

In addition to the strains requiring isoleucine and valine, a strain which

requires only valine has been used in this work. Strain 33051 requires valine for growth, and of the two isomers only $l(-)$-valine is active. Crosses of strain 33051 with a normal strain show segregation expected of a single gene difference.

An *isoleucineless* strain of *Escherichia coli* has also been used in these investigations. This strain, No. 58-336 (16), kindly made available to the author by Dr. E. L. Tatum, requires isoleucine for growth, and of the four isomeric isoleucines only $l(+)$-isoleucine is active.

Valine and Isoleucine Inhibitions—Valine alone will permit about one-half normal growth of strain 33051. Additions of isoleucine, however,
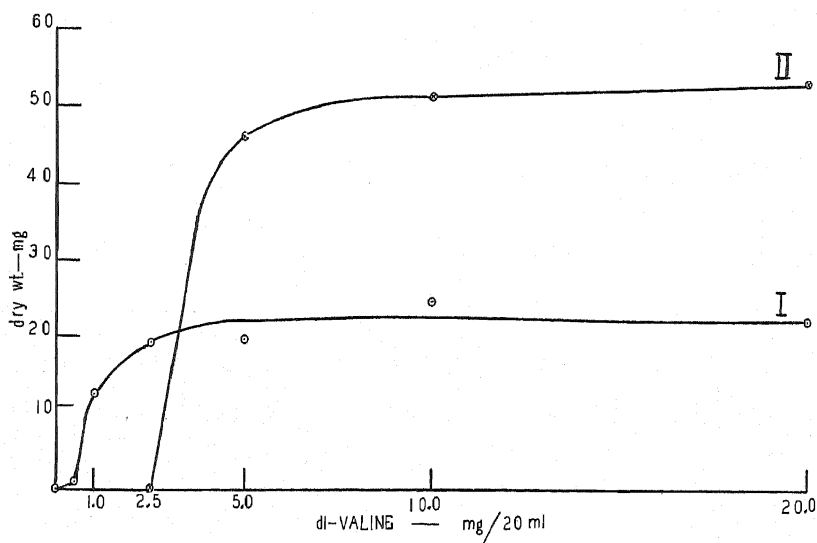
Fig. 2. Growth curve of strain 33051. Curve I, growth on valine; Curve II, growth on valine in the presence of 10 mg. per 20 ml. of *dl*-isoleucine.

bring the growth to normal (Fig. 2). Concentrations of isoleucine approximately 10 times that of valine completely inhibit growth (Fig. 2). The fact that in strain 33051 exogenous isoleucine appears to inhibit the utilization of exogenous valine suggests that similar inhibitions may be present in strain 16117.

Strain 16117 was originally observed to require a certain ratio of valine to isoleucine. The ratio giving maximum growth is 70 per cent $l(-)$-valine to 30 per cent $l(+)$-isoleucine (7). This ratio now appears to be due to a mutual inhibition of utilization of both amino acids. In the presence of adequate isoleucine and valine for growth, increasing valine concentrations give complete growth inhibition, which is overcome by increasing the

isoleucine concentration (Table I). Likewise, in the presence of a concentration of isoleucine and valine sufficient for growth, increasing isoleucine concentrations give complete growth inhibition, which is reversible by further valine additions (Table II).

These observations suggest that a genetic block of either isoleucine or valine synthesis would result in a double requirement. Externally supplied isoleucine would inhibit valine utilization in a mutant with a true isoleucine requirement, thus producing an apparent valine requirement. Or similarly a true valine requirement would produce an apparent isoleucine requirement. While this phenomenon is undoubtedly a contributing factor to the

TABLE I

Inhibition of Growth of Strain 16117 by dl-Valine

Growth is represented as mg. of mycelium, dry weight.

dl-Isoleucine, mg. per 20 ml.	*dl*-Valine, mg. per 20 ml.					
	0	1	2.5	5	10	20
0	0	0				0
1	0	29	38	9	1	0
20	0					36

TABLE II

Inhibition of Growth of Strain 16117 by dl-Isoleucine

Growth is represented as mg. of mycelium, dry weight.

dl-Valine, mg. per 20 ml.	*dl*-Isoleucine, mg. per 20 ml.				
	0	1.0	5.0	10.0	20.0
0	0	0			0
1	0	34	16	3.0	0
20	1.0	0	0	0	49

observed double requirement of strain 16117, it alone will not account for it, since strain 33051 will grow on valine alone. The growth stimulation or inhibition of strain 33051 caused by isoleucine additions to a valine-containing medium is likely due to the mutual isoleucine-valine inhibitions described for strain 16117. These inhibitions appear, therefore, to be not of sufficient magnitude to prevent ready detection of single requirements and would not account for the double requirement of strain 16117.

Synthetic Keto Acid Preparations—The original observations concerning the activity of keto acids on strain 16117 were made with impure keto acid preparations made by oxidation of racemic amino acids with hog *d*-amino oxidase (7). As a check on the results so obtained ketovaline and keto-isoleucine were prepared synthetically.

The previously reported inactivity of a mixture of two keto acids, and the activity of ketovaline, tested in the presence of isoleucine, were confirmed. The activity of ketoisoleucine preparations, made with d-amino oxidase and tested in the presence of valine, was rechecked and also confirmed. However, synthetic ketoisoleucine, tested under similar conditions, is totally inactive. In no instance has any activity of synthetic ketoisoleucine for growth of strain 16117 been observed. This failure of synthetic ketoisoleucine to support growth of strain 16117 is highly significant, and it is upon this observation that much of the scheme presented in Fig. 1 is based.

The inactivity of synthetic ketoisoleucine is not due to its racemic nature since d-amino oxidase oxidizes both $d(-)$-isoleucine and $d(-)$-alloisoleucine. Both the synthetic and d-amino oxidase preparations are therefore racemic.

Available evidence suggests that the ketoisoleucine of d-amino oxidase preparations is inactive for growth and that the observed activity of such

TABLE III

Activity of Ketovaline in Supporting Growth of Strain 16117

Growth is represented as mg. of mycelium, dry weight.

dl-Isoleucine, mg. per 20 ml.	Ketovaline, mg. per 20 ml.				
	0	0.5	1.0	2.0	4.0
0	0			0	
4	1.0	9	15	23	26

preparations is due to the presence of some additional substance. The chemical nature of this additional substance has not yet been determined.

Biosynthesis of Valine—The hydroxy acid analogue of valine can be used by strain 33051 for growth only after a lag period of several days, suggesting that it is not a normal intermediate in valine synthesis. Synthetic ketovaline, however, is active, and at low concentrations is more active than valine. This greater activity of the keto acid might be expected, since the phenomenon of inhibition of isoleucine utilization by exogenous valine is not present. At high concentrations (5 mg. per 20 ml.) the keto acid is less active than valine. That the keto acid is not more active than valine at higher concentrations is probably due to the inhibition of isoleucine synthesis by ketovaline, as discussed later. In accord with available evidence in other organisms (13, 14), the activity of ketovaline suggests that it may serve as an intermediate in valine synthesis in *Neurospora*. Strain 33051 is presumably genetically blocked, therefore, in a step involved in the formation of the carbon skeleton. As mentioned earlier, strain 16117 can use ketovaline for growth when tested in the presence of isoleucine (Table III)

but the maximum growth attained is usually about one-half that attained by normal strains. That strain 16117 grown on ketovaline does not attain normal growth is in agreement with the scheme presented in Fig. 1. The fact that strain 16117 can use ketovaline indicates that this strain can carry out the conversion of ketovaline to valine.

Biosynthesis of Isoleucine—In the hope that the biological activity of synthetic ketoisoleucine could be demonstrated tests with the *isoleucineless Escherichia coli* strain were carried out. The synthetic preparation of keto-isoleucine is active for growth of this mutant strain, indicating that growth inhibition by this keto acid preparation is of no great consequence. Keto-isoleucine appears to serve as an intermediate in isoleucine synthesis by this bacterium. This observation is in agreement with available evidence re-garding isoleucine and related amino acid syntheses in other organisms (13, 15). It is also of interest to note that a block in isoleucine synthesis in this mutant strain of *Escherichia coli* at a point prior to the conversion of keto-isoleucine to isoleucine does not lead to a double requirement. Strain 46003 of *Neurospora* is known to utilize either threonine or isoleucine for growth (H. J. Teas, unpublished). Ketoisoleucine was tested on this mutant and found active. This suggests that *Neurospora* can utilize keto-isoleucine. Strain 16117 cannot use ketoisoleucine for growth when tested in the presence of either valine or ketovaline. Since ketoisoleucine may serve as an intermediate in isoleucine synthesis in other organisms, and since *Neurospora* apparently can utilize it, strain 16117 is unable to carry out the amination of ketoisoleucine. The requirement for exogenous isoleu-cine appears, then, due to the inability of strain 16117 to convert ketoisoleu-cine to isoleucine. Strain 16117 is, therefore, known to be able to convert ketovaline to valine and unable to carry out the analogous reaction in isoleucine synthesis.

Keto Acid Inhibitions—Growth of normal *Escherichia coli* (K-12) is inhib-ited by valine or ketovaline, but not by isoleucine. The growth inhibitions caused by either valine or ketovaline can be overcome by isoleucine (E. L. Tatum, unpublished). The *isoleucineless Escherichia coli* strain discussed above shows no inhibition by valine in the presence of isoleucine. How-ever, at low concentrations, ketovaline completely inhibits the growth activity of ketoisoleucine (Table IV), this inhibition being overcome by isoleucine (Table IV). In the presence of isoleucine, ketovaline is only slightly inhibitory. These facts suggest that the inhibition by ketovaline is not an inhibition of the utilization of formed amino acid, but rather an inhibition of the conversion of keto acid to isoleucine.

Since ketovaline specifically inhibits the amination of ketoisoleucine in *Escherichia coli*, experiments were carried out to determine whether keto-isoleucine might not inhibit the amination of ketovaline in *Neurospora*.

This actually turns out to be the case. Ketoisoleucine, in concentrations equal to or exceeding the concentration of ketovaline, strongly inhibits the activity of ketovaline for growth of strain 16117 (Table V). However, in the presence of valine, ketoisoleucine shows no growth inhibition of strain 16117 (Table V). This indicates that the inhibition is in the conversion of

TABLE IV

Inhibition of Ketoisoleucine Growth Activity for Escherichia coli Strain 58-336 by Ketovaline

Growth is represented as optical density. Concentrations, mg. per 10 ml.

Ketoiso-leucine	Ketovaline										
	0		0.01			0.1			1.0		
	dl-Isoleucine		*dl*-Isoleucine			*dl*-Isoleucine			*dl*-Isoleucine		
	0	0.3	0	0.3	0.5	0	0.3	0.5	0	0.3	0.5
0	0	122	0	169		0	139		0	90	
0.05	80		0		123	0		143	0		118
0.3	106		94		104	6		104	0		88

TABLE V

Ketoisoleucine Inhibition of Ketovaline Activity on Growth of Strain 16117

Isoleucine	Valine	Ketoisoleucine	Ketovaline	Mycelium, dry weight
mg. per 20 ml.	*mg. per 20 ml.*	*mg. per 20 ml.*	*mg. per 20 ml.*	*mg.*
				0
2.0				1
	2.5			0
		5.0		0
			1.0	0
2.0			1.0	18
2.0		0.5	1.0	4
2.0		1.0	1.0	2
2.0		2.5	1.0	0
2.0		5.0	1.0	0
2.0		10.0	1.0	0
2.0	2.5	5.0	1.0	53
2.0	2.5	10.0	1.0	54

ketovaline to valine. A similar inhibiting effect of ketoisoleucine on the activity of ketovaline can be demonstrated in strain 33051. Here again the inhibition is caused by concentrations of ketoisoleucine equal to or exceeding the concentration of ketovaline, and the inhibition may be overcome by additions of valine.

The activity of the keto acid analogues of isoleucine and valine in sup-

porting growth of strain 16117 might, therefore, be summarized as follows: Ketoisoleucine is inactive either in combination with valine or ketovaline, indicating the inability of strain 16117 to carry out the conversion of keto-isoleucine to isoleucine. Ketovaline tested in the presence of isoleucine is active, indicating that the reactions involved in conversion of ketovaline to valine are operative in strain 16117. The conversion of ketovaline to valine, however, is strongly inhibited by the presence of exogenous keto-isoleucine. A summary of the growth response of the various strains to isoleucine, valine, ketoisoleucine, and ketovaline is shown in Table VI.

TABLE VI

Summary of Growth of Neurospora Strains 16117 and 33051 and Escherichia coli Strain 58-336 on Isoleucine (I), Valine (V), Ketoisoleucine (KI), Ketovaline (KV), and Various Combinations of These Four Compounds

I., inhibition; R., reversal.

Compound	Growth of		
	Strain 16117	Strain 33051	*Escherichia coli* 58-336
I..	−	−	+
V..	−	+	−
I + V...	+	+	+
KI...	−	−	+
KV...	−	+	−
KI + KV......................................	−	− I.	− I.
I + KV..	+	+	+
V + KI..	−	+	+
KI + KV + I.................................	− I.	− I.	+ R.
" + " + V....................................	−	+ R.	− I.
" + " + I + V...............................	+ R.	+	+

DISCUSSION

Available evidence suggests that single gene changes result in the impairment of but single enzymes (see the review by Beadle (1)). Mutant strains having a multiple growth factor requirement associated with a single gene change might, therefore, be expected, since many enzymes are known to act on a number of related substrates. A mutation affecting such an enzyme, would then be expected to cause a multiple deficiency by blocking comparable reactions in closely related compounds. The genetic block giving rise to the double requirement of strain 16117 was thought to be a mutation of this type (7). This interpretation, however, is no longer tenable, nor is the interpretation of a genetic block in the production of a precursor common to both isoleucine and valine.

Present evidence indicates that the conversion of ketoisoleucine to iso-

leucine is genetically blocked, while the comparable amination of ketovaline is not so blocked. If, therefore, the synthesis of valine is impaired by loss of an enzyme, it must be at some point prior to the formation of ketovaline, presumably in the synthesis of the characteristic carbon chain. If this were the case, the single enzyme lost in strain 16117 would be expected to catalyze normally two reactions of totally different character. As most, if not all enzymes have at least relative specificity, the suggestion of a primary genetic block in valine synthesis now seems unreasonable.

The evidence that exogenous ketoisoleucine specifically inhibits the amination of ketovaline provides the basis for a more plausible explanation of the double requirement of strain 16117. Investigations of this strain indicate that it is unable to convert ketoisoleucine to isoleucine, while it should synthesize it. Ketoisoleucine might, therefore, be expected to accumulate in the mycelium to a certain extent and would then block the amination of ketovaline. Accumulation of precursors prior to a genetic block have been demonstrated in *Neurospora* in a number of instances (6, 8, 17) and may be expected on theoretical grounds.

Preliminary attempts to demonstrate keto acid accumulation in the medium have not as yet been successful. However, only limited accumulation in the mycelium need occur to give the observed growth requirements of strain 16117. Strain 16117, in contrast to the normal strain of *Neurospora* or other mutant strains, however, does give rise to a characteristic odor when grown on isoleucine and valine, an odor resembling diacetyl. This may indicate that any excess keto acids formed are metabolized in a different way. Such side reactions of an accumulated intermediate have been observed in another instance (6).

The nature of the stimulating substance present in d-amino oxidase keto acid preparations and the accumulation of keto acids need to be demonstrated for confirmation of the present interpretation. However, all experimental results obtained to date are in agreement with the interpretation that the double requirement of strain 16117 represents a true genetic block in isoleucine synthesis and an apparent block in valine synthesis.

SUMMARY

Mutant *Neurospora* strain 16117, differing from normal by a single gene, requires the two amino acids isoleucine and valine for growth. Investigations as to the nature of this block are reported. This strain cannot use the keto acid analogue of isoleucine, prepared synthetically, in place of isoleucine, but it can use the keto acid analogue of valine. The keto acid analogue of isoleucine, however, specifically inhibits the conversion of α-ketoisovaleric acid to valine. It is therefore suggested that strain 16117 is genetically blocked in the conversion of α-keto-β-methyl-n-valeric acid

to isoleucine and that this genetic block results in an accumulation of this keto acid, which in turn inhibits the conversion of α-ketoisovaleric acid to valine. This genetic block in the synthesis of isoleucine would therefore give a strain with the apparent double requirement for isoleucine and valine of strain 16117.

BIBLIOGRAPHY

1. Beadle, G. W., *Chem. Rev.*, **37**, 15 (1945).
2. Beadle, G. W., *Physiol. Rev.*, **25**, 643 (1945).
3. Beadle, G. W., and Coornradt, V. L., *Genetics*, **29**, 291 (1944).
4. Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945).
5. Bonner, D., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **11**, in press.
6. Bonner, D., and Beadle, G. W., *Arch. Biochem.*, in press.
7. Bonner, D., Tatum, E. L., and Beadle, G. W., *Arch. Biochem.*, **3**, 71 (1943).
8. Horowitz, N. H., *J. Biol. Chem.*, **162**, 413 (1946).
9. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
10. Horowitz, N. H., Bonner, D., Mitchell, H. K., Tatum, E. L., and Beadle, G. W., *Am. Naturalist*, **79**, 304 (1945).
11. Mebus, A., *Monatsh. Chem.*, **26**, 485 (1905).
12. Rassow, B., and Bauer, R., *J. prakt. Chem.*, **80**, 97 (1909).
13. Rose, W. C., *Science*, **86**, 298 (1937).
14. Rose, W. C., Johnson, J. E., and Haines, W. J., *J. Biol. Chem.*, **145**, 679 (1942).
15. Schoenheimer, R., The dynamic state of body constituents, Cambridge (1942).
16. Tatum, E. L., *Proc. Nat. Acad. Sc.*, **31**, 215 (1945).
17. Tatum, E. L., Bonner, D., and Beadle, G. W., *Arch. Biochem.*, **3**, 477 (1944).

CRYSTALLINE PROTEIN WITH THYMONUCLEODEPOLY-MERASE ACTIVITY ISOLATED FROM BEEF PANCREAS*

By M. LASKOWSKI

(*From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee*)

The author believes that the proper name for this enzyme should be either desoxyribonucleinase (if the hydrolysis proceeds to the stage of mononucleotides) or desoxyribonuclease (if the hydrolysis stops on a higher level). Because, so far, no definite statement to this effect can be made, the provisional name is retained.

Crystalline protein was prepared by several similar procedures. The most convenient method so far found was the following. Fresh beef pancreas was collected in a slaughter-house and immediately immersed in cold 0.25 N sulfuric acid, as recommended by Kunitz and Northrop (1). The first three steps of preparation were identical with the method of McCarty (2).

Step 1—Extraction with 0.25 N sulfuric acid.

Step 2—Precipitation with ammonium sulfate between 0.2 and 0.4 saturation.

Step 3—Precipitation with ammonium sulfate between 0.17 and 0.3 saturation. The repetition of this step required by McCarty's method was omitted.

Step 4—The precipitate obtained in the previous step was collected on a Büchner funnel, with Whatman paper No. 50. It was mixed with 2 volumes of water and dialyzed in the cold against 0.01 N acetate buffer, pH 5, for at least 48 hours with frequent changes. The precipitate which formed was centrifuged off, washed with a small amount of 0.01 N acetate buffer, pH 5, and discarded (Fraction A).

Step 5—The combined liquid and wash fluid were treated with 0.5 volume of methyl alcohol, cooled previously to $-20°$. The mixture was left for 20 minutes in an ice bath, centrifuged in the cold, and the liquid was discarded.

Step 6—The precipitate (Fraction B) was redissolved in about one-fifth of the previous volume of water to which normal acetic acid was added dropwise until a complete solution of enzyme was achieved (pH 4.5 or somewhat lower). By careful addition of 0.1 N NaOH, the pH was brought

up until a trace of opalescence appeared (around pH 5). The mixture was left for 15 to 20 minutes at room temperature. If the first precipitate which formed did not show a typical silky appearance, it was centrifuged off and discarded. A few drops of 0.1 N NaOH were then added to the liquid until opalescence appeared. When the silkiness was noticed, the mixture was transferred to a refrigerator and left overnight. To complete the crystallization, 0.1 N NaOH was added dropwise, but not to exceed pH 5.5. Crystals thus obtained were plates (Fig. 1).
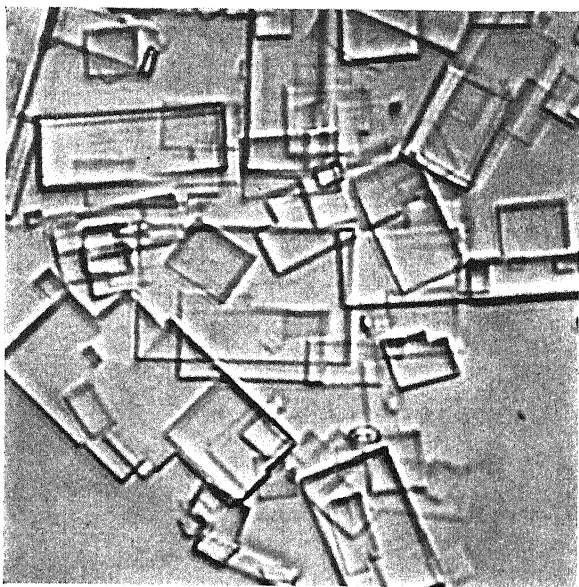
Fig. 1. Twice recrystallized protein, × 600

About one-half of the activity remained in the mother liquor. If the mother liquor was further treated with NaOH (pH over 5.5), an amorphous protein was precipitated. If the mother liquor was treated with methyl alcohol a few drops at a time at pH 5.5, crystals were obtained, but were found to be poorly shaped.[1]

[1] Several alternative procedures also led to crystalline preparation. One which did not involve methyl alcohol was the following: Step 4, the precipitate from Step 3 was dialyzed against 0.01 N acetate buffer at pH 4.7. The precipitate (Fraction A) was discarded. Step 5, the liquid from Step 4 was dialyzed against 0.01 N acetate buffer at pH 5. The precipitate (Fraction B) was saved. After repetition of dialysis against pH 4.7 and pH 5, a crystalline product was obtained from this fraction. Step 6, the liquid from Step 5 was dialyzed against acetate buffer at pH 5.5. The precipitate (Fraction C) was microcrystalline. After dissolving this precipitate in a small

Recrystallization was achieved by a repetition of Step 6, or else by dialyzing against 0.01 N acetate buffer, pH 4.5, and readjusting the pH until the first appearance of opalescence.

Not infrequently, during the recrystallization, the shape of the crystals was changed to long needles, resembling chymotrypsinogen (Fig. 2). In one case, both forms of crystals were present simultaneously. So far, it is not possible to define conditions specific for either of these crystalline forms.

Crystalline preparations thus obtained did not show a uniform activity (Table I). The following alternatives were therefore considered. (1) The
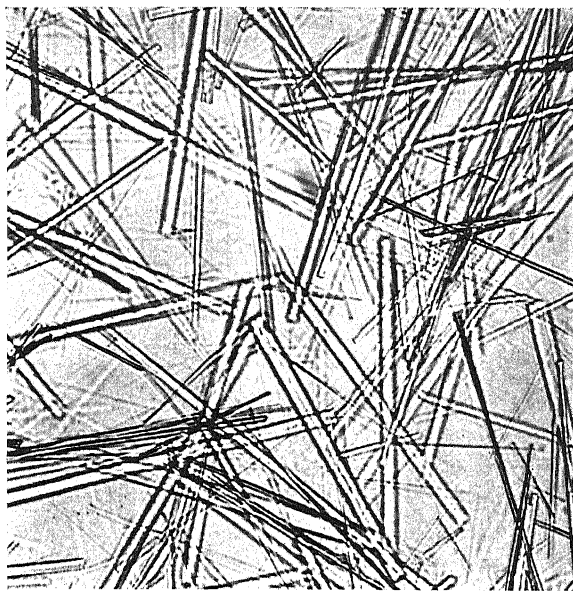
FIG. 2. Second crystalline form of the same protein as in Fig. 1. First recrystallization, × 300.

crystalline protein is not thymonucleodepolymerase, but is contaminated with a small amount of very active enzyme. (2) The crystalline protein is thymonucleodepolymerase, but, during the process of crystallization and subsequent storage, undergoes some minor structural changes resulting in the partial loss of activity. Such loss of activity may result either from the loss of a coenzyme (other than Mg), or the loss of an active group, such as —SH or S—S.

---

volume of dilute (0.01 N) acetic acid and readjusting the pH until the first appearance of opalescence, typical crystals were obtained. The loss of activity was, however, greater than in the method previously described.

Should the crystalline protein represent an inactive protein, its removal by crystallization should result in a considerable degree of purification of thymonucleodepolymerase in the mother liquor. This, however, was not the case. In the majority of cases the mother liquor showed only a slightly

TABLE I

Potencies of Different Crystalline Fractions of Thymonucleodepolymerase

Preparation No.	Crystallization	Potency, V units per mg. protein
Lyophilized enzyme prepared according to McCarty*		2,300
2. Ba	1st	25,000
Baa	2nd	16,000
Baaa	3rd	6,400
Baba	1st	35,000
3. C	1st	31,000
D2	1st	65,000
D2a	2nd	17,000
D2ba	1st	11,000
D2baa	2nd	3,000
4. B1	1st	2,400
5. B1	1st	1,500
B2	2nd	660

* Prior to lyophilizing, this preparation showed 10,000 V units per mg. of protein. Loss apparently occurred during this process.

TABLE II

Comparison of Potencies of Fractions from Which Crystalline Protein Was Obtained with Crystals and Mother Liquor

Potency is given as V units per mg. of protein.

Preparation No.	Prior to crystallization	Preparation No.	Crystals	Preparation No.	Mother liquor
2B	37,400	2Ba	25,600	2Bb	60,000
2Ba	25,600	2Baa	16,000	2Bab	19,400
2Baa	16,000	2Baaa	6,400	2Baab	10,000
2Bab	19,400	2Baba	35,000	2Babb	30,000
3C	30,000	3Ca	31,000	3Cb	37,000
4B	7,000	4Ba	2,400	4Bb	5,400

higher potency than the crystalline material (Table II). Furthermore, crystalline preparations were obtained, showing activities as high as 65,000 viscosity units per mg. of protein, only slightly lower in activity than any fraction so far obtained. In view of these facts, the first alternative was abandoned as untenable.

The search for a coenzyme led only to negative results. Combining the

crystalline fraction with the mother liquor did not increase the activity above the value of the sum of the two fractions. Addition of concentrated dialysate, or addition of freshly boiled pancreatic extract, was also ineffective.

The possibility that thymonucleodepolymerase requires for activity free —SH groups, and that these groups are oxidized during crystallization, was ruled out. The addition of hydrogen peroxide in concentration 10^{-3} M did not inhibit the action of thymonucleodepolymerase as measured either by V units (viscosity units (3)), or P units (acid-soluble phosphorus units (4)). Addition of either cyanide or cysteine in concentration 10^{-4} M to the solution of crystalline enzyme did not prevent the loss of activity during recrystallization.

The addition of either cysteine or cyanide produced an apparent enhancement of thymonucleodepolymerase activity as measured by V units (Table

TABLE III

Effect of Reducing Agents on Determination of Activity by Viscosity Method

	V units
Enzyme alone...	2.1
1.25×10^{-5} M cyanide alone............................	0.0
Enzyme + 1.25×10^{-5} M cyanide......................	4.0
Enzyme alone...	0.8
2.5×10^{-6} M cysteine alone............................	0.0
Enzyme + 2.5×10^{-6} M cysteine......................	1.6

III), while the same substances produced inhibition of thymonucleodepolymerase activity as measured by P units (Table IV). The apparent activation of enzyme found by viscosimetric methods is due to the effect of reducing substances on the substrate. With somewhat higher concentrations of reducing agents (Fig. 3), a gradual drop in viscosity of thymonucleic acid was observed which resembled the action of the enzyme.

The possibility of reducing active S—S linkages during the crystallization was also investigated. Addition of cystine to the reacting mixture showed no effect on V units and a slightly enhancing effect on P units (Table IV). Recrystallization in the presence of 10^{-4} M cystine or hydrogen peroxide did not prevent the loss of activity. The loss of activity during storage was, however, slowed down by the presence of hydrogen peroxide (Table V). The results of these experiments do not exclude the possibility that S—S linkage is required for the function of thymonucleodepolymerase. On the other hand, they indicate that not all of the loss of activity during the recrystallization and storage was due to the reduction of these linkages.

A series of experiments was performed in order to determine the optimal

conditions for storage of the purified material. The finding of McCarty (2) that 0.01 per cent gelatin exerts a protective action on very dilute solutions of thymonucleodepolymerase was confirmed (Table VI), although
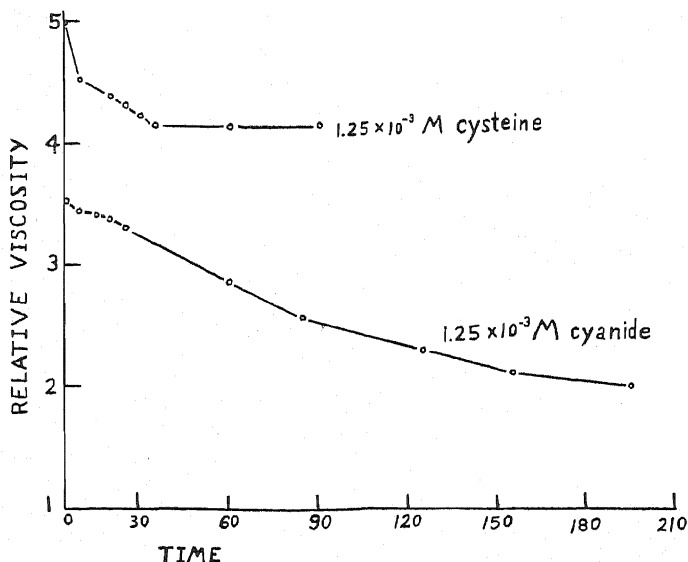
FIG. 3. Effect of cysteine and cyanide on the viscosity of thymonucleic acid. No enzyme added. All other conditions correspond to the conditions for the assay of thymonucleodepolymerase. Time in minutes.

TABLE IV

Effect of Some Substances on Determination of Activity of Thymonucleodepolymerase by Acid-Soluble Phosphorus Method

	P units
Enzyme alone	7.93
" + 1.25 × 10⁻³ M cyanide	5.20
" + 1.25 × 10⁻⁴ " "	6.05
" alone	9.7
" + 1.25 × 10⁻⁴ M cysteine	8.3
" alone	3.3
" + 1.25 × 10⁻⁴ M cystine	4.0
" alone	9.2
" + 1.25 × 10⁻⁴ M cystine	11.7

this protective action of gelatin was found to be of short duration. The inactivation in dilute solutions was much faster at pH 7 than below pH 4. The influence of pH on storage was also investigated, with somewhat less dilute solutions of crystalline enzyme (Fig. 4). Contrary to our previous

TABLE V

Effect of 10⁻³ M Hydrogen Peroxide on Storage of Crystalline Thymonucleodepolymerase; Preparation 4A4

	V units per cc.	V units per cc. after 1 mo.	Per cent activity recovered
No hydrogen peroxide........................	7200	880	12
10⁻³ M hydrogen peroxide added............	3100	1180	38

TABLE VI

Effect of Various Diluting Agents on Stability of Thymonucleodepolymerase

	Tested immediately after dilution		After 24 hrs. in cold	
	V units per sample	V units per cc. original	V units per sample	V units per cc. original
1% gelatin (1:10,000), 0.1 cc.	10.5	1,050,000		
1% " (1:1000), 0.1 "			0.9	9,000
Water (1:10,000), 0.1 cc.	2.0	200,000		
" (1:1000), 0.1 "			1.4	14,000
0.2 M borate buffer, pH 7 (1:10,000), 0.1 cc.	2.6	260,000		
0.2 M " " " 7 (1:1000), 0.1 cc.			0.0	
0.01 N acetic acid, (1:10,000), 0.1 cc.	2.9	290,000		
0.01 " " " (1:1000), 0.1 "			7.2	72,000
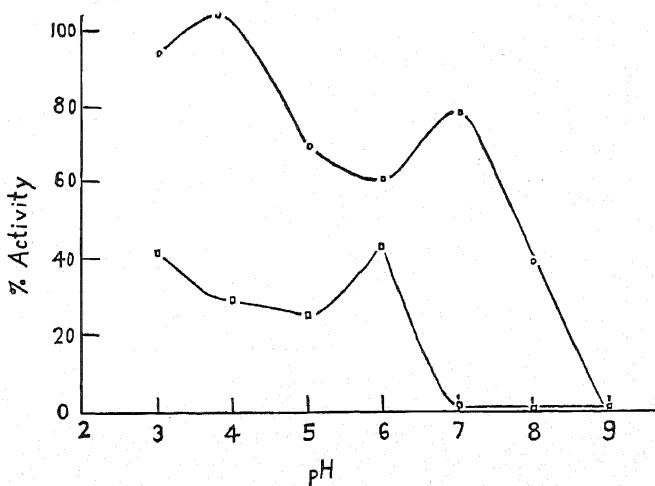
FIG. 4. Influence of pH on storage. All buffers are 0.1 M. pH 3, 4, 5, acetate buffers; pH 6, 7, phosphate buffers; pH 8, 9, borate buffers. Concentration of protein, 0.24 mg. per cc. Upper curve, after 24 hours in the refrigerator; lower curve, after the next 24 hours at room temperature.

observations on the crude enzyme (4), the purified enzyme was most stable in acid medium. The second optimum was probably accidental, since it shifted on 2 consecutive days. Storing the pure enzyme under 50 per cent saturated ammonium sulfate slightly decreased the speed of decomposition. The more concentrated the solution with respect to the enzyme, the smaller was the loss of activity during storage.

In the author's opinion, these results supply additional evidence in favor of the assumption that the crystalline protein is thymonucleodepolymerase, but that during the process of crystallization, and storage, some active groups (not yet identified) are lost, possibly as a result of hydrolysis, or reduction, or both.

DISCUSSION

We have previously noticed (4) that if thymonucleodepolymerase activity was tested by the two different methods (viscosity and acid-soluble phosphorus) the ratio between V units and P units varied comparatively little when the different stages of purification of the same batch of enzyme were compared. Variation was much greater when the corresponding stages of purification from different batches were compared. No explanation could have been offered at that time. The confusing findings concerning the influence of reducing agents on the determination of the activity of thymonucleodepolymerase by the two methods (Fig. 3, Tables III and IV), suggested at least a partial explanation for the variation in the V:P ratio. The excess of reducing groups (—SH) would result in an excessively high value for the V units and a low value for the P units. The observed variation, therefore, might have been due to the differences in concentration of reducing substances in different batches of enzyme.

Although the pure enzyme is extremely unstable in dilute solution, only highly dilute enzyme solutions can be used in our methods of testing (2). Both methods previously described by us (3, 4) should therefore be modified by the addition of gelatin during the enzyme incubations. In the experiments reported in this paper, activity tests were performed in the presence of 0.01 per cent gelatin.

Finally, a very important factor in determining the activity is the uniformity of the substrate. Although our substrate was prepared by the same method (Hammarsten (5)), striking differences in the values for V units occurred when the same enzyme was tested against different preparations of substrate. No explanation can be offered at present.

SUMMARY

A new crystalline protein from beef pancreas has been obtained and the method of preparing it has been described.

The freshly prepared protein showed high thymonucleodepolymerase activity, but recrystallization resulted in a considerable loss of activity.

Several possible causes of this loss were investigated and the tentative conclusion was reached that some active groups (not yet identified) are destroyed during the process of crystallization, possibly as a result of hydrolysis.

Evidence has been presented that thymonucleodepolymerase does not require intact —SH groups for activity. No definite statement can be made regarding S—S groups.

The conditions of assay of thymonucleodepolymerase have been discussed.

BIBLIOGRAPHY

1. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1936).
2. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
3. Laskowski, M., and Seidel, M. K., *Arch. Biochem.*, **7**, 465 (1945).
4. Laskowski, M., *Arch. Biochem.*, **11**, 41 (1946).
5. Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).

CHANGES IN THE ABSORPTION SPECTRA DUE TO AGING OF THE CARR-PRICE REACTION MIXTURE WITH VITAMIN A AND THE COMMON CAROTENOID PIGMENTS*

By M. J. CALDWELL† AND J. S. HUGHES

(From the Kansas Agricultural Experiment Station, Manhattan)

Carr and Price (1) in 1926 introduced a reagent for the quantitative estimation of vitamin A which was destined to play an important rôle in the study of the physiology of the vitamin. This reagent, a saturated solution of antimony trichloride in chloroform, still occupies a position of first importance in most laboratories where vitamin A is estimated by chemical means. The sensitivity of the reagent and the simplicity of its application have made its use in many cases the method of choice. However, its non-specificity and the transient character of the blue coloration produced with vitamin A have at times cast doubts on its general reliability.

Although numerous non-vitamin A substances are known to be chromogenic with antimony trichloride (2), difficulty is usually encountered only in the case of the carotenoid pigments which are closely related to vitamin A and which frequently accompany the vitamin in animal tissue. The colors produced by these pigments with the reagent display distinctly different absorption spectra and a much greater apparent stability than that due to vitamin A. The effect of increased temperature[1] and of intense illumination[2] on the instability of the Carr-Price colors is much more pronounced in the case of vitamin A than in the case of the carotenoids. The variation in stability has been made the basis for the differentiation of the vitamin A color from that of the other chromogens by several investigators (3–5).

Several workers have reported constants relating to the absorption spectra of the Carr-Price reaction products of vitamin A and of several of the other chromogenic substances. Gillam (6) has reported the wavelength of the absorption maxima and the corresponding $E_{1\,cm}^{1\%}$ values for β-carotene, lycopene, lutein, zeaxanthin, and vitamin A. Goldhammer and Kuen (7) have given corresponding values for carotene, xanthophyll, and for several of the sterols. Von Euler et al. (8) have published curves show-

---

[1] Caldwell, M. J., and Hughes, J. S., in preparation.

[2] Caldwell, M. J., and Hughes, J. S., in preparation.

ing absorption characteristics for β-carotene immediately after adding the reagent and also 30 minutes later.   Lamb, Mueller, and Beach (9) have published curves showing the antimony trichloride colors with ergosterol, cholesterol, and 7-dehydrocholesterol.   Gibson and Taylor (10) recently introduced a new technique for observing the rapidly changing spectra of the antimony trichloride-chromogen systems.   Their "dynamic method"
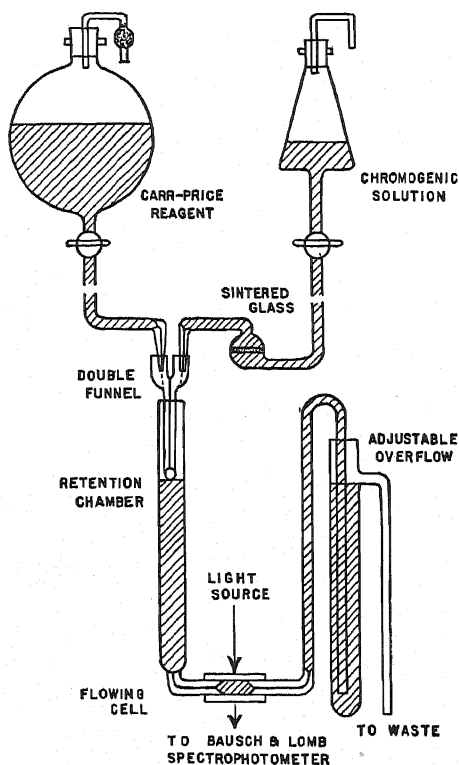
FIG. 1. Modified form of the flowing cell apparatus of Gibson and Taylor used in determining the absorption spectra by the "dynamic method."

involves the use of a "flowing cell" (Fig. 1) in which a steady state of flowing reagent and chromogen is maintained during the spectral measurement. These workers have presented curves for liver oil concentrates, their oxidized products, and for β-carotene, which show the rapid changes as the solution ages.

No comprehensive investigation has been found in the literature relating to the changing spectra of the antimony trichloride reaction products with vitamin A in its various forms and with the carotenoid pigments most likely

to interfere with the Carr-Price determination of vitamin A. It is the purpose of this paper to present these fundamental data which should prove of value in the further development of the Carr-Price method.

Procedure

Materials—The Carr-Price reagent was prepared by the general method of Koehn and Sherman (11). The antimony trichloride was dissolved in the purified chloroform at the rate of 22.5 gm. per 100 ml. of chloroform. The reagent was stored at room temperature in darkness or subdued light and filtered before use. Different batches of reagent exhibited great uniformity and stability.

Vitamin A[3] as the crystalline alcohol, crystalline acetate, and the liquid concentrate of the natural esters was available for the investigation. These preparations were preserved in the dark at −20° until ready for use. Stock solutions in U. S. P. chloroform were prepared and from these suitable dilutions ranging from 5 to 50 γ per ml. were made for use in the Carr-Price study.

The seven most common carotenoid pigments[4] listed by Zechmeister (12) were available for these studies. These were α-carotene, β-carotene, γ-carotene, lycopene, cryptoxanthin, lutein, and zeaxanthin. Although the quantity of γ-carotene available was insufficient for the study of its Carr-Price absorption spectrum, it was used in the other phases of the work to be reported. These pigments were also stored in the dark at −20° until used. Solutions in U. S. P. chloroform ranging in concentration from 100 to 400 γ per ml. were prepared, and the concentration and purity established in all cases by reference to the spectral data to be found in the literature (12, 13). The absorption data were obtained by the use of the Beckman spectrophotometer.

Apparatus and Methods—Most of the data here reported were obtained by use of the Beckman spectrophotometer. Before the spectral measurements were made the wave-length dial was set at the desired point and the instrument adjusted to zero optical density with a "blank" mixture of 1 ml. of chloroform and 9 ml. of the reagent. 1 ml. of the solution under test was placed in a lipped tube and 9 ml. of the reagent added. At this instant a stop-watch was started. The reaction mixture was transferred to the

[3] The vitamin A preparations were generously supplied by Dr. P. L. Harris of the Distillation Products, Inc.

[4] Dr. L. Zechmeister of the California Institute of Technology contributed samples of lycopene and zeaxanthin which were used in this study. Dr. John Porter of Purdue University and Dr. G. Mackinney of the University of California kindly supplied the lutein and the cryptoxanthin, respectively. The remaining carotenoid pigments were either prepared in this laboratory or purchased.

absorption cell, which was quickly placed in position for measurement in the Beckman spectrophotometer. At 30 seconds the first reading of optical density was taken, and readings were taken at intervals thereafter for a period of 10 minutes. This set of data represented the change in the optical density at the chosen wave-length, with time. The wave-length dial was reset, and the measurements repeated. This process was repeated at frequent intervals in the wave-length range from 500 to 700 mμ, well on either side of the 620 mμ maximum of the vitamin A Carr-Price reaction product. In plotting the data, all of the optical densities obtained at the same time after mixing were plotted against the wave-length. A series of absorption curves was thereby obtained, each member of which corresponds to a specific age of solution. These curves, viewed together, present a picture of the changing Carr-Price reaction product from 30 seconds to 10 minutes after mixing.

For time intervals less than 30 seconds recourse was had to the "dynamic method" of Gibson and Taylor, with a modified flowing cell apparatus. Fig. 1 shows the essential features of the flowing cell which was placed over the aperture of a visual Bausch and Lomb spectrophotometer. The reagent and chromogen solutions were allowed to mix at a constant rate and flow through the absorption cell at a time after mixing controlled by the level held in the retention chamber. When a steady state was reached, the absorption curve of the flowing mixture was determined in a normal fashion. The time after mixing was calculated by dividing the ml. of solution between the point of mixing and the center of the observation window by the rate of flow of the solution in ml. per minute. With the apparatus used, reproducible results could be obtained with mixtures from 2 to 30 seconds of age.

Calculations—All optical densities were converted to the corresponding $E_{1\,cm.}^{1\%}$ values by means of the Bouger-Beer (Beer-Lambert) law. Thus

$$E_{1\,cm.}^{1\%} = \frac{D}{c \cdot l}$$

where D is the observed optical density, c is the concentration in per cent of the chromogen, and l is the thickness of the absorption cell in centimeters.

DISCUSSION

The results obtained in this investigation are presented graphically in Figs. 2, 3, and 4. In these figures the $E_{1\,cm.}^{1\%}$ values are shown plotted against the wave-length, which extends well on either side of the 620 mμ maximum of the vitamin A Carr-Price reaction mixture. The age of the solutions measured from the instant of mixing is indicated on the various curves by numbers as well as by the coded lines. The solid lines represent

the solution of least age, while the dotted lines represent the oldest of the solutions.

Examination of the curves reveals marked differences between those relating to vitamin A in any of its forms and the curves of the carotenoid pigments. Vitamin A is seen to have chromogenic powers 10- to 25-fold
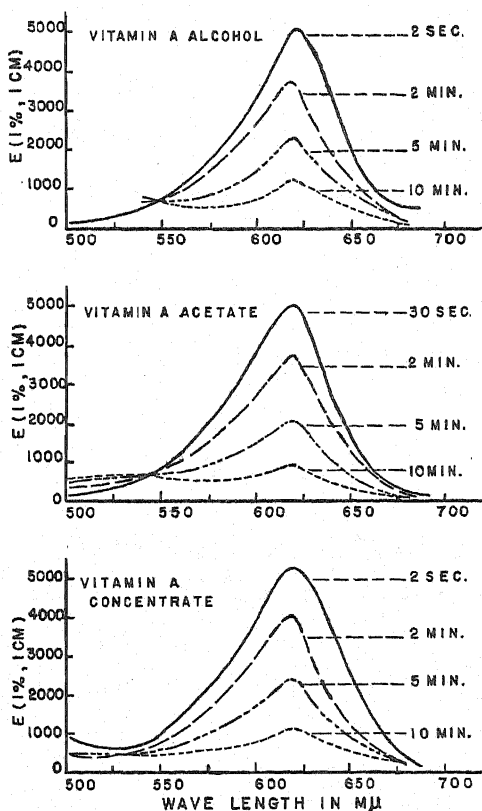
FIG. 2. Absorption spectra of the Carr-Price reaction products with vitamin A as the alcohol, acetate, and the concentrate of the natural esters, at various times after mixing. The E (1%, 1 cm.) values are calculated in terms of the alcohol equivalent.

greater than the common carotenoids in the region of the vitamin A maxima. Vitamin A, as alcohol, acetate, or natural ester, is unique among the chromogens in possessing a single strong absorption band (maximum at 620 mμ) which rapidly decreases with time. Changes of a more complex nature are exhibited by β-carotene, lutein, and zeaxanthin. Here the absorption maxima shift as a wave or in steps toward the red end of the spectrum as the solution ages. The remaining chromogens, α-carotene,

lycopene, and cryptoxanthin, show relatively simple absorption spectra, which gradually rise or fall with time. In no case can the Carr-Price color be termed "stable."

A study of these absorption curves makes clear the differences in the "stability" of the Carr-Price colors, as normally measured with a filter colorimeter or a diffraction instrument of equally wide wave band. In the
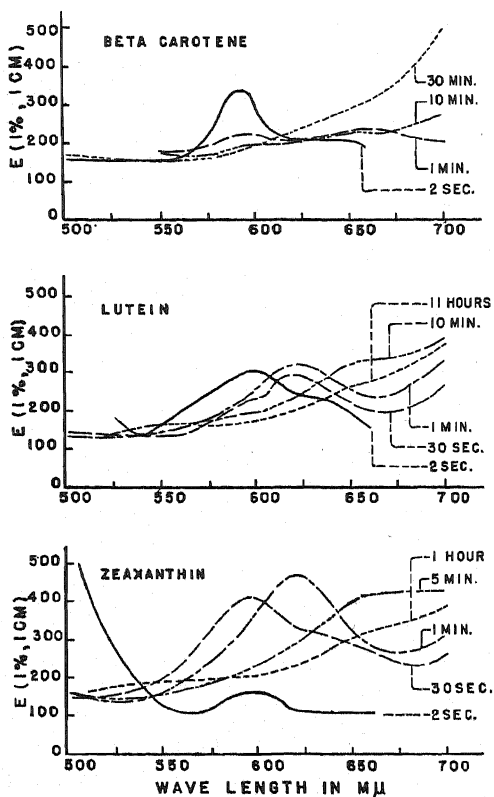
FIG. 3. Absorption spectra of the Carr-Price reaction products with β-carotene, lutein, and zeaxanthin at various times after mixing of the reactants.

case of vitamin A, only the decreasing absorption in the region of 620 mμ is recorded, while in the case of the carotenoids major changes occurring in the character of the absorption spectra may be entirely overlooked, due to the width of the filter band used in making the measurement. Thus, the Carr-Price colors with the various carotenoid pigments may be recorded as stable, increasing, or decreasing, depending on the integrated area under the absorption curves between the limits imposed by the optical system involved in the measurement.

SUMMARY

1. Spectral absorption curves for the Carr-Price reaction mixture with vitamin A as alcohol, acetate, and concentrate of the natural esters have been presented for reaction mixtures of ages varying from 2 seconds to 10 minutes.
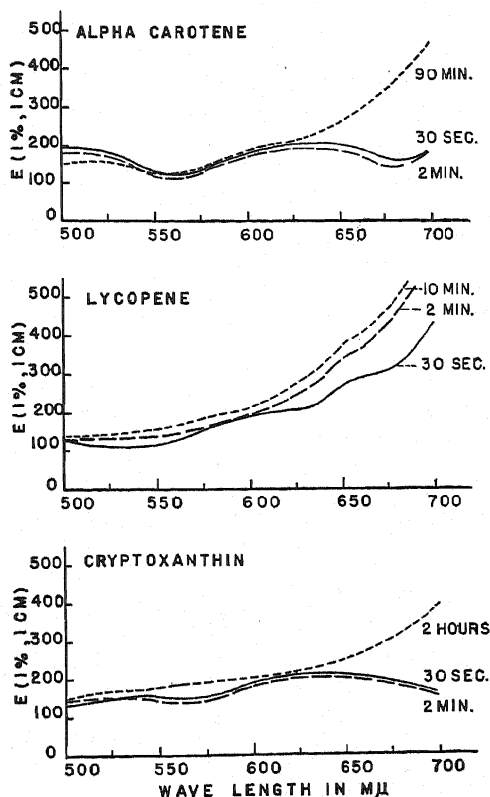
Fig. 4. Absorption spectra of the Carr-Price reaction products with α-carotene, lycopene, and cryptoxanthin at various times after mixing of the reactants.

2. Similar data have been presented for six of the common carotenoid pigments, α-carotene, β-carotene, lycopene, cryptoxanthin, lutein, and zeaxanthin.

BIBLIOGRAPHY

1. Carr, F. H., and Price, E. A., *Biochem. J.*, **20**, 497 (1926).
2. Delaby, R., Sabetay, S., and Janot, M., *Compt. rend. Acad.*, **198**, 276 (1934).
3. Oser, B. L., Melnick, D., and Pader, M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 724 (1943).
4. Brew, W., and Scott, M. B., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 46 (1946).
5. Meunier, P., and Raoul, Y., *Compt. rend. Acad.*, **206**, 1148 (1938).
6. Gillam, A. E., *Biochem. J.*, **29**, 1831 (1935).
7. Goldhammer, H., and Kuen, F. M., *Biochem. Z.*, **267**, 406 (1934).
8. von Euler, B., von Euler, H., and Hellstrom, H., *Biochem. Z.*, **203**, 370 (1928).
9. Lamb, F. W., Mueller, A., and Beach, G. W., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 187 (1946).
10. Gibson, G. P., and Taylor, R. J., *Analyst*, **70**, 449 (1945).
11. Koehn, C. J., and Sherman, W. C., *J. Biol. Chem.*, **132**, 527 (1940).
12. Zechmeister, L., *Chem. Rev.*, **34**, 267 (1944).
13. Zscheile, F. P., White, J. W., Jr., Beadle, B. W., and Roach, J. R., *Plant Physiol.*, **17**, 331 (1942).

THE EFFECT OF TRYPTOPHANE ON THE URINARY EXCRETION OF NICOTINIC ACID IN RATS*

By S. A. SINGAL, A. P. BRIGGS, V. P. SYDENSTRICKER, AND JULIA M. LITTLEJOHN

(From the Departments of Biochemistry and Medicine, University of Georgia School of Medicine, Augusta)

Krehl *et al.* (1, 2) have observed a growth retardation in rats when large amounts of corn grits were included in a low protein ration. The addition of either $l(-)$-tryptophane or nicotinic acid corrected the deficiency and allowed normal growth to proceed. The beneficial action of tryptophane has also been observed in nicotinic acid deficiency in the chick (3) and the mouse (4). Although recent results (5) indicate that corn is not an essential dietary component for the production of the deficiency state in the rat, a pellagragenic agent in corn has been reported for the mouse (6).

In order to elucidate the interchangeable rôle of tryptophane and nicotinic acid, we have studied the effect of tryptophane and other substances on the urinary excretion of nicotinic acid derivatives in rats on corn and non-corn rations. The results of these experiments are reported here.

EXPERIMENTAL

Wistar rats, 20 days old, were divided into groups of two and placed in metabolism cages. After 2 days on commercial animal ration, they were given the experimental diets *ad libitum*. The composition of the synthetic diet was casein (Labco) 15, sucrose 74, cottonseed oil 3, salts[1] 4, cystine 0.15, cod liver oil 2, and sodium chloride 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 2.0 mg., riboflavin 2.0 mg., pyridoxine 2.0 mg., calcium pantothenate 4.0 mg., and choline chloride 1.0 gm. The basal ration was prepared by mixing 60 parts of the above diet with 40 parts of corn grits. Supplements of tryptophane and casein replaced an equal amount of sucrose.

The 24 hour urine specimens were initially collected in 7 per cent HCl and usually diluted to 100 ml. with distilled water. The hydrolysis of urine was accomplished by autoclaving at 15 pounds for 15 minutes 2.0

[1] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 109, 657 (1935).

ml. of diluted urine with an equal volume of either 2.0 N H_2SO_4 or NaOH. The nicotinic acid content of the urine specimens and hydrolysates was determined microbiologically by the method of Snell and Wright (7) as modified by Isbell (8) and chemically by the method previously employed (9), as were the total methylated derivatives of nicotinic acid. 24 hour fecal specimens were prepared for microbiological assay by autoclaving the

TABLE I

Urinary Excretion of Nicotinic Acid in Rats

All data are expressed in micrograms per 24 hours per pair of rats.

Days	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
0	70	51	43	35	44	40	47
1	114	16	15	20	79	16	156
2	212	17	16	14	97	16	524
3	358	20	22	18	186	15	393
5	155	28	18	14	118	14	600
9	237	47	18	16	165	16	390
14	217	39	23	16	171	18	566
22	280	65	20		257	38	675
28	389	66	27	28	281	51	
29			30	30			
30			51	54			
31			37	152			
33			48	217			
35			46	165			
41			58	296			
46			55	299			
52			95	352			

Group I, basal diet + 0.5 per cent $l(-)$-tryptophane; Group II, basal + 1.0 mg. per cent of nicotinic acid; Group III, basal; Group IV, basal; Group V, basal + 0.5 per cent dl-tryptophane; Group VI, basal + 11 per cent casein; Group VII, basal + 1.0 per cent dl-tryptophane.

Beginning with the 29th day the basal diet of Groups III and IV was supplemented with 1.0 mg. per cent of nicotinic acid and 0.5 per cent $l(-)$-tryptophane respectively.

finely ground material with 1.0 N H_2SO_4 for 15 minutes. The extracts were neutralized hot to pH 4.5 with brom-cresol green as indicator, cooled to room temperature, filtered, and neutralized to pH 6.8 with brom-thymol blue as indicator.

From the results presented in Table I it is evident that the inclusion of $l(-)$-tryptophane or the racemic mixture in the basal ration results in an immediate increase in urinary nicotinic acid within 24 hours, reaching a maximum several days later. Nicotinic acid or supplementary casein was

without effect in this respect, although these supplements permitted normal growth when added to the basal diet. However, after an initial decrease (1 to 9 days) upon institution of the experimental diets, the urinary nicotinic acid increased very gradually, which may be an indication of normal growth. The maintenance of a low value in rats on the basal diet appears characteristic of the deficiency state. It is interesting to note that in Group IV the addition of tryptophane to the diet on the 29th day of deficiency results in an increased excretion of nicotinic acid, but only after a delay of 2 days

TABLE II

Fecal Excretion of Nicotinic Acid in Rats

All data are expressed in micrograms per 24 hours per pair of rats. Group diets are as given in Table I.

Days	Group I	Group II	Group III	Group IV	Group V	Group VI
0	104	91	113	122	123	130
1	47	51	44	97	118	76
2	27	19	32	51	63	65
3	32	22	16	44	41	32
5	26	31	21	27	56	17
9	47	45	22	32	47	45
14	72	47	26	17		57
22	95	65	32	20	75	84
28	106	95	27	23	94	126
29			27	30		
30			39	39		
31			62	87		
33			66	57		
35			75	88		
41			56			
46			76	100		
52			85	103		

There is no reflection in the fecal excretion of the relatively large amounts of urinary nicotinic acid excreted after tryptophane (Table II). Presumably, nicotinic acid of bacterial origin, like that derived from diet, would be reflected in the urinary fraction of methylated derivatives (10) and not in the free nicotinic acid fraction, which was observed to increase here. In addition, the rapid response of the urinary nicotinic acid to the administration and withdrawal of tryptophane (Tables III and IV) suggests a direct, rather than an indirect origin.

Tryptophane, when acid-hydrolyzed alone or with normal rat urine, failed to influence the nicotinic acid values obtained in assay. However, when a urine sample of a rat receiving tryptophane was similarly hydro-

lyzed the value increased from 358 to 770 γ on the basis of a 24 hour period. Similar results were obtained in other groups receiving tryptophane. Autoclaving these specimens at pH 6.8 or in 1.0 N NaOH was without effect in this respect. Since nicotinic acid and its amide have equal activities for the assay organism *Lactobacillus arabinosus* and nicotinuric acid does not require preliminary hydrolysis to show the same activity as its theoretical equivalent of nicotinic acid (7), it would appear that following the administration of tryptophane there is excreted a nicotinic acid precursor, which after acid hydrolysis can replace nicotinic acid as a growth substance for the assay organism.[2]

In Table III are shown the results of the administration of *dl*-tryptophane to mature rats maintained on commercial animal food. To avoid any deleterious effect of an acid preservative upon the estimation of the nico-

TABLE III

Effect of dl-Tryptophane on Urinary Excretion of Nicotinic Acid in Rats

All data are expressed in micrograms per 24 hours per pair of rats, each of which received by stomach tube 200 mg. of *dl*-tryptophane the 3rd and 4th days.

Days	Nicotinic acid*		
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis
1	69	104	79
2	78	143 (201)	96
3	197	1175	254
4	202	3124 (2710)	286
5	45	88	74

* Values in parentheses were obtained by chemical assay.

tinic acid precursor, toluene was employed in this and subsequent experiments. It is apparent that after tryptophane rats on non-corn rations also excrete a larger amount of nicotinic acid and, in addition, a much larger quantity of the nicotinic acid precursor, as measured by the difference in values obtained by acid and alkaline hydrolysis. A similar increase in the cyanogen bromide-metol reaction for nicotinic acid is observed after acid hydrolysis.

Because *dl*-tryptophane at the level administered (400 mg. per pair) resulted in a toxic manifestation of reduced food intake,[3] the experiment

[2] Studies in this laboratory indicate that N-methylnicotinamide or its acid hydrolysate cannot replace the nicotinic acid requirement of *Lactobacillus arabinosus*.
[3] The daily food intake per pair of mature rats was reduced from 28 gm. of commercial animal ration to 13 gm., when this diet was supplemented with 2.0 per cent *dl*-tryptophane. When *l*(−)-tryptophane was employed, this reduction in food intake was not observed.

was repeated by employing the natural isomer (Table IV). The results here are similar to those of the previous experiment, but do show much

TABLE IV

Effect of $l(-)$-Tryptophane on Urinary Excretion of Nicotinic Acid Derivatives in Rats

All data for nicotinic acid are expressed in micrograms per 24 hours per pair of rats.

Days	Tryptophane ingested per 24 hrs. per pair	Nicotinic acid			Methylated derivatives*
		No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	mg.				
1	0	87	122	104	830
2	0	103	142	114	990
3	0	96	122	112	640
4	440	472	4917	505	1320
5	500	709	5155	957	2090
6	440	682	4675	705	2080
7	480	829	3555	787	2580
8	520	632	3377	640	2320
9	520	742	2955	713	2570
10	0	230	428	241	1800
11	0	92	122	109	1180
12	0	93	127	105	680

* Obtained by chemical assay.

TABLE V

Separation of Nicotinic Acid Precursor with Norit A from Urine of Rats Receiving $l(-)$-Tryptophane

	Nicotinic acid*		
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis
	γ	γ	γ
Sample..........................	633	3377	640
Norit A filtrate..................	244	340	275
" " eluate...................	67 (40)	2470 (2475)	83

* Values in parentheses were obtained by chemical assay.

larger excretion values for both nicotinic acid and the precursor.[4, 5] Methylated derivatives, which include the biochemically important N-methyl-

[4] Alkaline treatment as such does not destroy the nicotinic acid precursor, for a "tryptophane" urine specimen containing 8.5 and 26 γ of nicotinic acid after alkaline and acid hydrolysis, respectively, when subjected to successive alkaline and acid hydrolysis contained 28 γ.

[5] Detectable quantities of the precursor have not been found in liver extract powder, yeast, or peptone or in cultures of *Escherichia coli*, *Staphylococcus aureus*, or *Lactobacillus arabinosus* 17-5.

nicotinamide (11, 12), are also substantially increased. The withdrawal of the supplementary tryptophane results in an immediate decrease in the excretion of all of the above derivatives, reaching preexperimental control levels by 48 hours.

In elaborating procedures for the isolation of the nicotinic acid precursor, its adsorption behavior has been studied. It is adsorbed almost completely by Darco G-60, Lloyd's reagent, and norit A from urine made 0.1 N with HCl and is eluted by 0.1 N NaOH from these materials with recoveries of 43, 50, and 87 per cent respectively. 77 per cent of the precursor is removed by Decalso, but elution with alkali is poor. Zinc hydroxide removes only a small quantity of the substance. In addition to the larger recoveries with norit A, only a small amount of the free nicotinic acid adsorbed from urine is eluted under these conditions (Table V).

DISCUSSION

Studies on nicotinic acid balance (10) and tissues (13) have already indicated that the rat can synthesize nicotinic acid. Although the means by which this was accomplished were unknown, the synthesis appeared related to the protein level of the diet. An extra dietary source of nicotinic acid in the human has also been suggested (14).

The increased excretion of nicotinic acid derivatives in rats receiving tryptophane suggests that this amino acid may be important in this synthesis. Similar results have been observed by Rosen et al. (15). It may appear that the effect of supplementary tryptophane observed by Krehl et al. (1, 2) and the absence of a nicotinic acid requirement in the rat on diets containing adequate amounts of protein (13) are, in effect, related to this synthesis.

It has been suggested that the tryptophane requirement of the rat is related to the nicotinic acid content of the diet (5). The synthesis of nicotinic acid from tryptophane similarly suggests that the nicotinic acid requirement is related to the amount of the amino acid available. It has been shown that the nicotinic acid requirement of the chick is abolished by the presence of adequate amounts of tryptophane (3) and the results in the pig (16) indicate a similar action by the protein level of the diet. Perhaps the interchangeable rôle of tryptophane and nicotinic acid finds an analogy in the methionine-cystine relationship (17), in that the rat's requirement for both tryptophane and nicotinic acid can be met by either adequate amounts of the amino acid alone or a minimal amount of the amino acid supplemented with nicotinic acid.

The appearance of an acid-labile nicotinic acid precursor in the urine of rats receiving tryptophane suggests that it may be an intermediate in the synthesis of nicotinic acid. Its production in the presence of large amounts of tryptophane, as employed here, may well exceed the rat's capacity for

converting it to nicotinic acid. However, the possibility exists that it may represent only a by-product in the metabolism of tryptophane unrelated to the synthesis of nicotinic acid. It is, of interest, to recall that Koser *et al.* (18) observed that quinolinic acid by heat treatment is decarboxylated to nicotinic acid, which was then available as a growth factor for the dysentery bacilli. This dicarboxylic acid derivative of pyridine does resemble the behavior of the nicotinic acid precursor with regard to heat treatment in acid, alkali, and at pH 6.8. Although concentrates of the latter prepared from urine show a positive $FeSO_4$ reaction for quinolinic acid (19), the nature of this reaction is not known and the identity of the urinary substance must await its isolation and characterization. The formation of the quinoline ring from the indole nucleus does occur in the synthesis of kynurenic acid (20).

SUMMARY

It has been observed that rats receiving tryptophane excrete relatively large amounts of nicotinic acid, a methylated derivative of nicotinic acid, and an unidentified substance which is converted to nicotinic acid by acid, but not alkaline, hydrolysis. The latter substance is excreted in largest quantities at the level of the amino acid fed. Nicotinic acid and supple-mentary casein are without effect in this respect.

BIBLIOGRAPHY

1. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101**, 283 (1945).
2. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101**, 489 (1945).
3. Briggs, G. M., *J. Biol. Chem.*, **161**, 749 (1945).
4. Woolley, D. W., *J. Biol. Chem.*, **162**, 179 (1946).
5. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 403 (1946).
6. Woolley, D. W., *J. Biol. Chem.*, **163**, 773 (1946).
7. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
8. Isbell, H., *J. Biol. Chem.*, **144**, 567 (1942).
9. Briggs, A. P., Singal, S. A., and Sydenstricker, V. P., *J. Nutr.*, **29**, 331 (1945).
10. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **142**, 401 (1942).
11. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395 (1943).
12. Najjar, V. A., White, V., and Scott, D. B. M., *Bull. Johns Hopkins Hosp.*, **74**, 378 (1944).
13. Dann, W. J., *J. Biol. Chem.*, **141**, 803 (1941).
14. Ellinger, P., and Coulson, R. A., *Biochem. J.*, **38**, 265 (1944).
15. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **163**, 344 (1946).
16. Wintrobe, M. M., Stein, H. J., Follis, R. H., Jr., and Humphreys, S., *J. Nutr.*, **30**, 395 (1945).
17. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).
18. Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, **43**, 391 (1940).
19. Koenigs, W., *Ber. chem. Ges.*, **12**, 983 (1877).
20. Kotake, Y., in Annual review of biochemistry, Stanford University, **4**, 225 (1935).

CONVERSION OF TRIGONELLINE TO NICOTINIC ACID

By JESSE W. HUFF*

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

In 1940 Sarett, Perlzweig, and Levy (1, 2) developed a method for the quantitative estimation of trigonelline. This method was based upon the observation that, when trigonelline was moderately heated with strong alkali in the presence of ammonia, a substance was produced which gave the König color reaction, with cyanogen bromide and an aromatic amine, under the conditions in which nicotinic acid or the amide reacted most specifically. With very small amounts of trigonelline not exceeding 0.2 mg. the yield of this substance was constant, equivalent to 70 per cent in terms of nicotinic acid, and gave proportional values. The authors assumed that the pyridine ring of the trigonelline was split with the elimination of methylamine and that the ring was closed with ammonia to yield nicotinic acid. The isolation of this substance and its identification as nicotinic acid were hampered by the low yield, 1 to 5 per cent, with samples of trigonelline greater than 0.5 mg. and by the production of a large excess of colored products.

From a consideration of the reactions probably involved in this transformation (see the diagram) it was possible to devise a method whereby yields of sufficient amounts of nicotinic acid were obtained to permit isolation and identification.

Trigonelline has previously been converted to nicotinic acid by methods which involved a direct removal of the methyl group without breaking the ring. Jahns (3) has reported that trigonelline is converted to nicotinic acid and methyl chloride by heating with hydrochloric acid in a closed tube at a temperature of over 260°. Weijlard, Tishler, and Messerly (4) have recently achieved a cleavage by a transmethylation. Trigonelline hydrate and pyridine hydrochloride, when heated at 200°, yielded nicotinic acid and N-methylpyridinium chloride.

By analogy with the work of Decker and Kaufmann (5) on the effect of alkali on N-methylpyridinium salts, a quaternary pyridinium base (II), first formed from the salt (I), rearranges into the α-carbinol (III). The α-carbinol is extremely reactive and is easily split by heating with

alkali. Hantzsch (6) has shown that heating with alkali splits off methyl-amine from trigonelline and leaves a substituted glutaconic aldehyde derivative (IV) which rapidly undergoes other reactions.

Complex-substituted pyridine derivatives are prepared by treating various substituted glutaconic aldehydes with ammonia to close the ring (7). By analogy the glutaconic aldehyde derivative (II) obtained from trigonelline would be expected to yield nicotinic acid (V). The action of potassium hydroxide and ammonia on trigonelline in the method of Perlzweig et al. (1, 2) to yield a nicotinic acid-like substance is then a combination of two reactions, splitting of the N-substituted ring with alkali and subsequent closure of the ring with ammonia to produce a ring
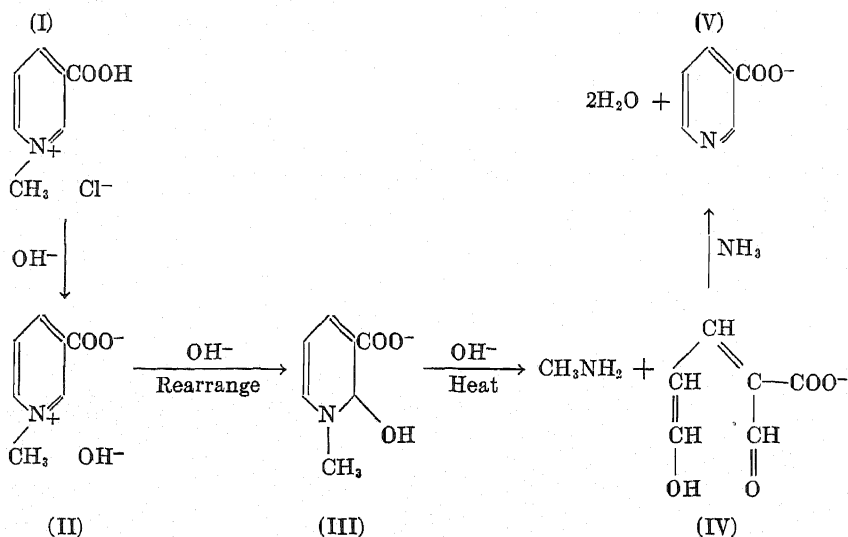
with the nitrogen in its tertiary state. The amount of nicotinic acid obtained from the glutaconic aldehyde produced by splitting of trigonelline would depend upon the result of a competition between ring closure with ammonia and the other reactions of which the reactive aldehyde is capable.

Under the conditions employed in the reaction it has been found that the splitting of the ring of trigonelline and the polymerization or oxidation of the aldehyde derivative thus formed are fairly rapid reactions, whereas the ring closure with ammonia is relatively slow. With the small amounts (10 to 200 γ) of trigonelline employed in the quantitative method (1, 2) the amount of the aldehyde present is small, and in view of the high concentration of ammonia initially present, the mass law favors the ring closure

in spite of its slow rate. However, when large amounts of trigonelline are used, very high concentrations of the aldehyde, much of which is converted to other substances with consequent lowering of the yield of the nicotinic acid-like substance, are reached. It is thus obvious that in order to obtain good yields of nicotinic acid the concentration of the glutaconic aldehyde derivative must be kept low. This can be obtained in either of two ways, (a) by carrying out the reaction in a large volume of alkali or (b) by using a small volume and adding the trigonelline in small portions over a period of several hours.

EXPERIMENTAL

The latter method appeared to be technically more feasible, and was tested experimentally as follows. 50 ml. of 6 N KOH, 1 ml. of concentrated ammonium hydroxide solution, and 1.5 gm. of urea were measured into a 200 ml. Grignard flask equipped with a stirrer and immersed in a 75–80° bath. When the contents of the flask had reached the temperature of the bath, a solution of 12 N KOH and an aqueous solution containing 66.6 mg. of trigonelline hydrochloride per ml. were added simultaneously from two burettes at the rate of 0.5 ml. per hour. A total of 800 mg. of the trigonelline hydrochloride was added in 24 hours. The heating was continued for 2 hours after the last addition. After this time nitrogen was bubbled through the hot solution to remove the ammonia. A quantitative determination for nicotinic acid on the solution at this point, employing the cyanogen bromide method (2), indicated that 172 mg. of nicotinic acid have been produced. This represents a yield of 30.5 per cent.[1]

The light yellow solution was neutralized by the addition of about 24 ml. of 18 N H_2SO_4. The K_2SO_4 which crystallized out of solution during the neutralization was removed by filtration, washed several times with ice water, and discarded. The yellow solution (volume 200 ml.) was rendered strongly acid (pH <1) by the addition of 13 ml. of 18 N H_2SO_4 and extracted with ether for 20 hours in a continuous extractor. The ether extract contained about 7 mg. of nicotinic acid plus 200 mg. of a white crystalline organic acid which was not identified at this time. If this organic acid were not removed at this point, it would subsequently be extracted along with the nicotinic acid and render the crystallization of the latter more difficult. At the low pH employed in the above ether extraction the nicotinic acid present as sulfate was not readily taken up by the ether.

The aqueous solution remaining from the extraction was adjusted to

[1] When 300 mg. of trigonelline hydrochloride were added in one portion to 50 ml. of 6 N KOH containing urea and NH_3 and the solution allowed to stand at 75° for 24 hours, there was obtained a yield of only 6 per cent of nicotinic acid.

pH 3.0 by the addition of strong alkali and again extracted with ether for 20 hours. The ether extract contained by direct measurement 160 mg. of nicotinic acid. The ether was evaporated to dryness; the residue was recrystallized from ethanol and yielded 125 mg. of small needle-shaped crystals. The melting point after recrystallization from water was 234–236° (uncorrected). The mixed melting point with pure nicotinic acid was unchanged. A sample of the isolated material and nicotinic acid, when measured by the colorimetric method (2), gave the same amount of color. The L values in the Evelyn colorimeter per microgram were 0.0226 and 0.0227 respectively.

SUMMARY

A procedure is presented whereby the yield of the nicotinic acid-like substance, produced from trigonelline by heating with potassium hydroxide ammonia, is increased to a degree permitting isolation. The substance was isolated from the reaction mixture and identified as nicotinic acid. A theoretical consideration of the reactions involved is presented.

BIBLIOGRAPHY

1. Sarett, H. P., Perlzweig, W. A., and Levy, E. D., *J. Biol. Chem.*, **135**, 483 (1940).
2. Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, **136**, 729 (1940).
3. Jahns, E., *Ber. chem. Ges.*, **20**, 2840 (1887).
4. Weijlard, J., Tishler, M., and Messerly, J. P., *J. Am. Chem. Soc.*, **66**, 1319 (1944).
5. Decker, E., and Kaufmann, A., *J. prakt. Chem.*, **84**, 432 (1911).
6. Hantzsch, A., *Ber. chem. Ges.*, **19**, 31 (1886).
7. Maier-Bode, H., and Altpeter, J., Das Pyridin und seine Derivate in Wissenschaft und Technik, Halle-on-the-Salle (1934).

THE RÔLE OF ARGININE IN GROWTH WITH SOME
OBSERVATIONS ON THE EFFECTS OF
ARGININIC ACID*

By ALECK BORMAN, THOMAS R. WOOD, HOWARD C. BLACK, ELEANOR
G. ANDERSON, M. JANE OESTERLING, MADELYN WOMACK,
AND WILLIAM C. ROSE†

*(From the Division of Biochemistry, Noyes Laboratory of Chemistry,
University of Illinois, Urbana)*

In previous papers from this laboratory consideration has been given to
the nutritive rôle of eighteen amino acids (*cf.* Rose and Fierke (11)). Of
these, nine were shown to be essential dietary components for the rat, and
nine were classified as non-essential in the sense that they can be synthe-
sized by the organism.

Four amino acids which are generally recognized as components of pro-
teins remain to be classified with respect to their growth effects in the
rat.[1] These are arginine, proline, hydroxyproline, and glutamic acid.
The earlier literature dealing with these amino acids has been reviewed
elsewhere (Rose (10)), and need not be repeated here. It is sufficient to
point out that as early as 1930 Scull and Rose (13) demonstrated the syn-
thesis of arginine *in vivo*, and that most investigators have regarded proline,
hydroxyproline, and glutamic acid as non-essential constituents of the
food. It should be borne in mind, however, that earlier reports dealt with
the effects of diets containing hydrolyzed proteins from which the amino
acids in question had been removed as completely as available methods
permitted. Under these conditions traces may have remained in the
hydrolysates. In view of this possibility, we stated several years ago that
"final judgment must await the results of feeding experiments involving the
use of mixtures of highly purified amino acids" (St. Julian and Rose (15)).
The present paper describes experiments of this nature. The results

[1] This series of papers was interrupted by the war and the extra duties of the senior
author resulting therefrom.

demonstrate that arginine exerts a growth-stimulatory action. This was contrary to our expectations in view of the previous observations of Scull and Rose. That this conflict is apparent rather than real will be made clear below.

Inasmuch as animals can grow even when arginine is absent from the food, an unusually large number of experiments was necessary in order to establish the magnitude of the growth stimulation exerted by this amino acid, and to avoid the possibility that conclusions might be based on fortuitous results. To conserve space, the earlier tests will be summarized quite briefly; and only in the more recent investigations, in which the quality of the diets has been improved, will the details of the methods be recorded.

Invariably, the amino acids which served as the nitrogenous components of the food were shown to be analytically pure. Furthermore, those which had been prepared from proteins were tested for arginine by the Weber (16) modification of the Sakaguchi (12) procedure. This test is applicable in the presence of most other amino acids. Tyrosine and tryptophane tend to induce atypical reactions, and traces of arginine cannot be detected in the presence of considerable quantities of histidine. In order to exclude the possibility that our histidine might have been contaminated with small amounts of arginine, the former, in several instances, was recrystallized five times after it yielded correct analytical values. This did not alter the intensity of the arginine stimulation.

The first experiment involved a comparison of the growth effects of two diets differing from each other in that one contained arginine, glutamic acid, and the prolines. The results showed that the animals which received these amino acids succeeded in making better gains than did their litter mates which were deprived of them. Since no attempt was made to equalize the nitrogen content of the two rations, the possibility existed that this inequality might have been responsible for the difference in growth behavior of the two groups.

In a second test the diets furnished identical quantities of nitrogen, and the amino acids were added individually. Thus, one group of animals received arginine, another glutamic acid, a third proline, and a fourth hydroxyproline. The data pointed to arginine as the effective agent. A slight stimulation appeared to have been induced by proline, but the effects were so small as to be regarded as insignificant at the time. In the light of the more recent observation of Stetten and Schoenheimer (14) that proline "labeled" with deuterium and N^{15} is converted into hydroxyproline, glutamic acid, and ornithine, the effect on growth of each of these 5-carbon

compounds requires further investigation. Such experiments are already in progress in this laboratory. The present paper is concerned with the rôle of arginine.

In view of the apparent growth-stimulatory activity of arginine observed in the preliminary experiments mentioned above,[2] a large number of additional tests have been conducted. These may be grouped conveniently into three series. Series I involved the use of diets containing mixtures of eighteen amino acids with and without the addition of arginine. The mixtures were devoid of norleucine, hydroxyglutamic acid, and citrulline. Norleucine is unnecessary for growth (cf. Womack and Rose (17)) and in our hands has proved to be moderately detrimental (Rose and Fierke, unpublished data). Furthermore, the status of this amino acid (Consden et al. (1)) and of hydroxyglutamic acid (Nicolet and Shinn (8)) as components of proteins has been questioned in recent years. Citrulline is non-essential (cf. Rose and Fierke (11)). The possibility of its replacing arginine for growth purposes will be considered in another paper. The amino acid mixtures in this series contained 0.2 per cent of $l(-)$-proline, 0.1 per cent of $l(-)$-hydroxyproline, and 2.0 per cent of $l(+)$-glutamic acid. Thus, if one or more of these amino acids is convertible into arginine, at a rate commensurate with the arginine requirement for maximum growth, one would not anticipate that the addition of arginine would exert a stimulatory effect.

Thirty-two litters of young rats were used in Series I. Each litter was divided as equitably as possible, with respect to the body weight and the sex of the numbers, into two groups. One group received the basal arginine-free ration. The other group received the basal diet supplemented with 0.6 or 0.7 per cent of $l(+)$-arginine monohydrochloride. The animals were housed separately, and were permitted to ingest the diets ad libitum for 28 days. The water containers were replenished daily and the food cups as required.

Of the 102 animals which received the arginine-free diet the mean gain was 37.7 ± 0.44 gm. Of the 108 animals which received the same basal ration supplemented with arginine the mean gain was 46.2 ± 0.52 gm. The mean difference, 8.5 gm., would appear to indicate unmistakably that arginine exerts a stimulatory effect upon growth even when the food contains small amounts of proline, hydroxyproline, and glutamic acid. This conclusion is substantiated by the fact that the ratio of the mean difference to the probable error of the difference is 12.5. Generally, ratios of 4 or more are regarded as definitely significant. A ratio of 12.5 implies that the

[2] These preliminary tests were carried out by Edwin T. Mertz and J. Kenneth Gunther.

odds against the difference being due to chance alone are overwhelming (approximately 10^{17} to 1).

Series II involved the use of several basal diets, all of which contained mixtures of the nine amino acids previously shown to be essential for the growth of the rat. Thus, all diets were devoid of the prolines and glutamic acid, and differed from each other only in minor details. Seven of the amino acids were synthetic products, while three, namely tryptophane, histidine monohydrochloride, and arginine monohydrochloride, were derived from proteins. In several experiments, both the tryptophane and the histidine monohydrochloride were recrystallized five times after they yielded correct analytical values in order to exclude possible contamination with arginine.

Eighteen litters, totaling 99 animals, were employed in Series II. Of these, forty-nine rats received the basal rations without arginine, and 50 the same rations supplemented with 0.24 per cent of $l(+)$-arginine monohydrochloride (equivalent to 0.2 per cent of the free amino acid).[3] The duration of the tests, as in Series I, was 28 days.

The mean gain of the animals which ingested the arginine-free diet was 28.9 ± 0.41 gm. The mean gain of the animals which ingested the ration supplemented with arginine was 36.0 ± 0.55 gm. Thus, the mean difference in favor of the rats which received arginine amounted to 7.1 gm. The ratio of the mean difference to the probable error of the difference was 10.3. This implies that the odds against the occurrence of a ratio as great or greater than this, due to chance alone, are in excess of 6.5×10^{10} to 1.

The above data leave little room for doubt that arginine exerts a growth-stimulatory action in the rat. This conclusion has received further confirmation in recent experiments involving the use of an improved basal diet. For several years, systematic investigations in this laboratory have been directed toward the establishment of the most favorable conditions for the growth of animals on diets containing mixtures of amino acids. These studies appear to indicate that rats make somewhat better gains when the rations contain relatively small proportions of fats. In most of our previous experiments the diets have contained in excess of 30 per cent of fat. Furthermore, the salt mixture of Osborne and Mendel (9) formerly employed by us does not furnish sufficient phosphorus to permit *maximum* gains unless the diet contains casein, yeast, or some other additional source of this element (*cf.* Jones and Foster (6)). We now use Mixture 12 of Jones and Foster (6). Finally, in all of our earlier amino acid studies the vitamin intakes of the animals were suboptimal. This fact was thoroughly appreciated at the time, but could not be avoided without introducing into the

---

[3] Unpublished experiments have shown that, under the conditions of our tests, larger percentages of arginine are not more effective.

food too much nitrogen of an unknown nature. With the increase in knowledge of the vitamin requirements of the rat and the advent of abundant supplies of the crystalline vitamins, this unavoidable defect in our previous practice has been remedied.

It seemed likely that a diet which permits relatively rapid growth, when all required amino acids are included, might be particularly well adapted to

TABLE I

Composition of Amino Acid Mixture

	Mixture XXIII-a	
	Physiologically active	As used
	gm.	gm.
Glycine...	0.10	0.10
Alanine..	0.20	0.40*
Serine...	0.10	0.20*
Valine...	1.00	2.00*
Leucine..	1.20	2.40*
Isoleucine.....................................	0.80	1.60*
Cystine..	0.20	0.20
Methionine....................................	0.80	0.80*
Threonine.....................................	0.70	1.40*
Phenylalanine.................................	1.20	1.20*
Tyrosine.......................................	0.60	0.60
Proline..	0.20	0.20
Hydroxyproline...............................	0.10	0.10
Tryptophane...................................	0.40	0.40
Aspartic acid..................................	0.20	0.20
Glutamic "	2.00	2.00
Lysine	1.20	
" monohydrochloride......................		3.00*
Histidine......................................	0.70	
" monohydrochloride monohydrate........		0.95
Sodium bicarbonate...........................		1.76
	11.70	19.51

* Racemic acids.

a study of the arginine effect. With this consideration in mind, additional experiments were undertaken. Simultaneously, the possible replacement of arginine for growth purposes by its α-hydroxy analogue, argininic acid, was tested. These experiments constitute Series III. As in the investigations of Series I and II, the test period was 28 days and the animals were permitted to ingest the food *ad libitum*.

The composition of the amino acid mixture (Mixture XXIII-a) is shown in Table I. As will be observed, it was devoid of arginine. This amino

acid (or argininic acid) was incorporated in the food as desired. Nine of the amino acids were racemic products. In order to allow for the unnatural enantiomorphs, twice the desired quantities were included in the mixture except in the case of phenylalanine and methionine. Previous studies have shown that both forms of these amino acids are equally effective for the growth of the rat (*cf.* Rose (10)).

The make-up of the diets is summarized in Tables II and III. Diet 1 was devoid of arginine and argininic acid. Diet 2 contained 0.24 per cent of *l*(+)-arginine monohydrochloride. Diet 3 was supplemented with 0.4

TABLE II

Composition of Diets

	Diet 1	Diet 2	Diet 3
	gm.	*gm.*	*gm.*
Amino acid Mixture XXIII-a...............	19.51	19.51	19.51
l(+)-Arginine monohydrochloride...........	0	0.24	0
l(−)-Argininic acid.........................	0	0	0.40
Sucrose...................................	71.74	71.50	71.34
Cellu flour................................	2.00	2.00	2.00
Salt mixture*..............................	4.00	4.00	4.00
Corn oil...................................	2.00	2.00	2.00
Haliver oil†...............................	0.05	0.05	0.05
Inositol...................................	0.10	0.10	0.10
Choline chloride...........................	0.20	0.20	0.20
Liver extract‡.............................	0.40	0.40	0.40
	100.00	100.00	100.00

* Jones and Foster (6).

† This contained 65,000 U. S. P. units of vitamin A and 10,000 U. S. P. units of vitamin D per gm.

‡ Wilson's liver powder 1:20.

per cent, or two equivalents, of *l*(−)-argininic acid. The quantities of vitamins listed in Table III were admixed thoroughly with 1 kilo of each of the diets recorded in Table II. In our hands, better growth is obtained when the vitamins are included in the diets than when they are administered separately. The unknown nitrogen present in the liver extract could not have exceeded 32 mg. per 100 gm. of food, and consequently could not have contributed significant quantities of arginine.

Argininic acid was prepared by a slight modification of the procedure of Felix and Schneider (4). The yield of pure product from 40 gm. of *l*(+)-arginine monohydrochloride was 22 gm., or 67 per cent of the theory. The compound showed a specific rotation in aqueous solution of −11.85°. This

is identical with the value reported by Hunter and Woodward (5). Felix and Müller (3) reported a specific rotation of $-12.5°$. Analysis yielded the following results.

$$C_6H_{13}N_3O_3. \quad \text{Calculated.} \quad \text{C } 41.11, \text{ H } 7.48, \text{ N } 24.00$$
$$\text{Found.} \quad \text{``} \ 41.08, \text{``} \ 7.46, \text{``} \ 23.84$$

It is well known that the deamination of an amino acid with nitrous acid, as in the above procedure, is not accompanied by a Walden inversion, and that the hydroxy acid so formed has the configuration characteristic of the amino acid from which it is derived (cf. Levene (7)). Thus, the product obtained in the above reaction is correctly designated as $l(-)$-argininic acid.

TABLE III

Vitamin Supplements

	Added to each kilo of the rations
	mg.
Thiamine hydrochloride.............................	5
Riboflavin..	10
Pyridoxine hydrochloride............................	5
Nicotinic acid....................................	5
Calcium d-pantothenate............................	25
p-Aminobenzoic acid..............................	300
α-Tocopherol................................	25
2-Methyl-1,4-naphthoquinone.......................	2
	γ
Biotin*..	100

* S. M. A. Corporation concentrate equivalent to 100 γ of pure biotin.

Three litters of rats were used in the feeding trials. Each litter was divided as equitably as possible among the three diets. The results are summarized in Table IV. The mean gain of the eight animals which were deprived of arginine was 39.0 gm., while the corresponding gain of the ten animals which received arginine was 72.6 gm. Thus, with diets of otherwise high quality the rats deprived of dietary arginine gained scarcely 54 per cent as much as did their litter mates which received this amino acid. If one examines the behavior of each sex separately, it is noted that without and with arginine the males gained 42.0 and 82.0 gm. and the females 36.0 and 63.2 gm. respectively. *In the light of the highly significant findings in the animals of Series I and II, the marked differences now observed following the use of an improved basal ration provide convincing proof of the rôle of arginine as a growth factor in the rat.*

The present findings are not in conflict with the observation of Scull and Rose (13) that arginine can be synthesized by the rat. Indeed, the results of the two investigations confirm each other in a remarkable and unexpected fashion. If arginine could not be synthesized *in vivo*, animals deprived of

TABLE IV

Effects of Arginine and Argininic Acid on Growth

The experiments covered 28 days each.

Litter No.	Rat No. and sex	Total increase in weight	Total food intake	Supplement
		gm.	*gm.*	
1	6242 ♂	40	190	None
	6243 ♀	35	181	"
	6244 ♀	33	176	"
	6245 ♀	47	213	Argininic acid
	6246 ♂	46	240*	" "
	6247 ♀	43	213*	" "
	6248 ♂	90	286	Arginine
	6249 ♀	66	276*	"
	6250 ♀	58	243*	"
2	6251 ♂	50	199	None
	6252 ♀	40	174	"
	6253 ♂	47	191	Argininic acid
	6254 ♂	51	187	" "
	6255 ♀	42	189	" "
	6256 ♀	42	198*	" "
	6257 ♂	76	236	Arginine
	6258 ♂	84	252	"
	6259 ♀	64	237*	"
	6260 ♀	65	242	"
3	6320 ♂	37	193	None
	6321 ♂	41	193	"
	6322 ♀	36	195	"
	6323 ♂	49	206*	Argininic acid
	6324 ♂	60	250*	" "
	6325 ♀	45	229	" "
	6326 ♂	80	242	Arginine
	6327 ♂	80	250	"
	6328 ♀	63	258*	"

* The animal scattered food; the recorded intake is probably too high.

it would necessarily *lose* weight, just as they do when they are denied access to tryptophane, leucine, or any other dietary essential. The fact that growth occurs is proof that the amino acid is manufactured in the organism. The important point is that its synthesis does not keep pace with the needs of the animal for *normal* growth. This differentiates arginine from the non-

essential amino acids, which apparently can be produced in sufficient amounts to meet fully the requirements of the animal.

The classification of arginine as dispensable or indispensable is purely a matter of definition. At the present time, we define an indispensable dietary component as one which cannot be synthesized by the animal organism, out of materials *ordinarily available* to the cells (*cf.* Cox and Rose (2)), at a speed commensurate with the demands for *normal* growth. Under this definition, arginine must be classified as indispensable, although it alone of its group may be excluded from the food without occasioning a *loss* in weight. As additional information comes to light concerning the rôle of the amino acids in other functions such as reproduction and detoxication, redefinition of the term "indispensable" may become necessary. As currently used in this laboratory it refers to growth alone. Furthermore, one must always denote the species in classifying a dietary component as dispensable or indispensable since important species differences have already been recognized and doubtless are destined to become more numerous.

With respect to argininic acid, the data in Table IV demonstrate that it is much less effective than arginine. The mean gain of the ten rats which received argininic acid was 47.2 gm. as compared to 39.0 gm. for the animals which ingested the arginine-free diet. An examination of the behavior of each sex shows that without any supplement and with argininic acid the males gained 42.0 and 50.6 gm., and the females 36.0 and 43.8 gm. respectively. Evidently some growth stimulation occurred; but certainly argininic acid is a poor substitute for arginine. One should recall that two equivalents of argininic acid were present in Diet 3. Had 50 per cent been converted into arginine, growth comparable to that induced by the amino acid should have occurred. As a matter of fact, the mean growth increment in the rats which received the hydroxy analogue was only about one-fourth that observed in the arginine controls. Despite the hazard of basing calculations upon so few data, one is tempted to conclude that not more than one-eighth, or 12 per cent, of the argininic acid was transformed into arginine. Even less evidence of growth stimulation by argininic acid was observed in a group of animals (the details are not included in this paper) which received a basal diet containing nine instead of eighteen amino acids. In these tests, supplementation with argininic acid occasioned, in twenty-six rats, only about one-sixth of the mean growth increment observed in twenty-three animals receiving arginine. This would appear to indicate that not more than 8 per cent of the argininic acid was aminated.

SUMMARY

Investigations upon more than 300 young rats receiving diets containing mixtures of amino acids in place of proteins have demonstrated that in this

species arginine is a necessary dietary component for *optimum* growth. The effects of arginine are highly significant statistically whether the basal diet contains nine or eighteen amino acids. Evidently, the synthesis of arginine *in vivo* does not keep pace with the arginine requirements of the cells for *maximum* increases in weight.

Argininic acid, when added to an arginine-free diet, exerts only a moderate effect on the growth rate of young rats. This indicates that the hydroxy compound is not readily transformed into the amino acid by the species in question.

BIBLIOGRAPHY

1. Consden, R., Gordon, A. H., Martin, A. J. P., Rosenheim, O., and Synge, R. L. M., *Biochem. J.*, **39**, 251 (1945).
2. Cox, G. J., and Rose, W. C., *J. Biol. Chem.*, **68**, 781 (1926).
3. Felix, K., and Müller, H., *Z. physiol. Chem.*, **174**, 112 (1928).
4. Felix, K., and Schneider, H., *Z. physiol. Chem.*, **255**, 132 (1938).
5. Hunter, A., and Woodward, H. E., *Biochem. J.*, **35**, 1298 (1941).
6. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
7. Levene, P. A., *Chem. Rev.*, **2**, 179 (1926).
8. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **142**, 139 (1942).
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
10. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).
11. Rose, W. C., and Fierke, S. S., *J. Biol. Chem.*, **143**, 115 (1942).
12. Sakaguchi, S., *J. Biochem.*, **5**, 25 (1925).
13. Scull, C. W., and Rose, W. C., *J. Biol. Chem.*, **89**, 109 (1930).
14. Stetten, M. R., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 113 (1944).
15. St. Julian, R. R., and Rose, W. C., *J. Biol. Chem.*, **98**, 457 (1932).
16. Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930).
17. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **116**, 381 (1936).

PHOSPHORUS COMPOUNDS IN ANIMAL TISSUES

V. THE PRECIPITATION OF NUCLEOPROTEINS FROM RAT LIVER HOMOGENATES BY CALCIUM CHLORIDE

By WALTER C. SCHNEIDER*

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison) .

The precipitation of nucleoproteins by dilute solutions of calcium chloride and other inorganic salts was discovered by Huiskamp (3) about 50 years ago, but little information has been gained about the quantitative aspects of the precipitation or of the relative precipitabilities of the pentose as compared to the desoxypentose nucleoproteins. As a result of the development of methods for the quantitative extraction of the nucleic acids from animal tissue suspensions and for their estimation by colorimetric reactions (5), a study of these problems has been greatly facilitated. The present report describes an investigation of the precipitation of the nucleoproteins of rat liver homogenates by calcium chloride solutions.

Materials and Methods

Tissue Preparations—The liver was removed as rapidly as possible from rats killed by decapitation and was homogenized in ice-cold distilled water in the apparatus of Potter and Elvehjem (4). The liver homogenate was either used as such or was freed of nuclei and mitochondria by centrifuging the homogenate as described previously (6).

Measurement of Nucleic Acids—Desoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA) were extracted as described in an earlier paper after acid-soluble compounds had been removed (5). DNA and PNA were determined in the nucleic acid extract by the diphenylamine and orcinol reactions, respectively (5).

Measurement of Protein—Protein was determined by the colorimetric method described by Weichselbaum (7). Crystalline bovine albumin was used as a standard. The results are reported in terms of mg. of protein. Since turbidities were encountered in the application of the method to liver homogenates and liver extracts, the protein results reported are to be considered only as relative values and not as the absolute protein content of the material in question.

Results

When calcium chloride is added to a liver homogenate in an appropriate concentration, a finely divided nucleoprotein precipitate immediately forms. The precipitate centrifuges down at relatively low speeds (2000 R.P.M.) and the slightly opalescent supernatant can be poured off without disturbing the precipitate.   The following experiments were designed to test the effectiveness of the precipitation and the influence of different factors on the precipitation.
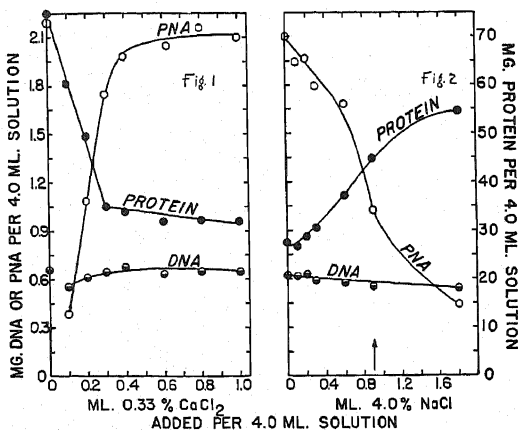
FIG. 1. (Left) The effect of CaCl₂ concentration on the precipitation of nucleoproteins from a homogenate of rat liver in distilled water.   The precipitations were carried out in centrifuge tubes and the final volume in each tube was 4.0 ml.   Each tube contained the equivalent of 250 mg. of fresh rat liver in the form of a homogenate. CaCl₂ was added as indicated in the chart and, after mixing, the contents of the tubes were centrifuged for 10 minutes at 600g to remove the precipitated nucleoprotein. The supernatants were poured off and the precipitates were washed with 4.0 ml. of a solution of the same CaCl₂ concentrations which had been used in the precipitations. PNA and DNA were determined in the precipitates and protein was estimated in the supernatants from the CaCl₂ precipitates.   The amounts of PNA, DNA, and protein found in the original homogenates are given at zero CaCl₂ concentration.

FIG. 2. (Right) The effect of NaCl concentration on the precipitation of nucleoproteins by CaCl₂.   The final volume and the tissue concentration were the same as in Fig. 1.   NaCl was added in the amounts indicated (the arrow indicates 0.9 per cent NaCl) and, after mixing, 0.1 ml. of 2.2 per cent CaCl₂ was added to each tube.   The contents of the tubes were centrifuged as in Fig. 1.   PNA and DNA were determined on the CaCl₂ precipitates and protein was estimated in the supernatants from the precipitates.

Effect of Calcium Chloride Concentration—The effect of the CaCl₂ concentration on the precipitation of the nucleoproteins of a rat liver homogenate is given in Fig. 1, which shows that at calcium concentrations greater than 0.033 per cent the nucleic acids were found almost entirely in the

precipitate produced by the calcium. At lower levels of CaCl₂, the amount of PNA found in the precipitate varied directly with the calcium chloride concentration. On the other hand, the amount of protein found in the supernatant from the calcium chloride precipitate varied inversely with the calcium ion concentration. Thus the precipitation of PNA was apparently correlated with precipitation of protein from the homogenate. The DNA was found entirely in the sediment at all calcium concentrations, presumably because the DNA was present in intact nuclei which were centrifuged down regardless of the calcium concentration.

Effect of NaCl Concentration on Precipitation of Rat Liver Nucleoproteins by CaCl₂—Attempts to precipitate nucleoproteins with CaCl₂ from liver homogenates in isotonic NaCl led to low yields of PNA in the precipitate. Fig. 2 shows the effect of different levels of NaCl on the precipitation of nucleoproteins by 0.055 per cent CaCl₂. Fig. 2 shows that, as the NaCl concentration was increased, the amount of PNA in the precipitate decreased, while the amount of protein in the supernatant from the CaCl₂ precipitate increased. The entire amount of DNA present in the original homogenate was found in the precipitate at all levels of NaCl. Thus the NaCl apparently had a solubilizing action on the CaCl₂-pentose nucleoprotein precipitate. This view was supported by experiments in which the levels of CaCl₂ added in the presence of isotonic NaCl were increased to 0.275 per cent. At this level of CaCl₂, more pentose nucleoprotein was precipitated than at 0.055 per cent, but the precipitation of nucleoprotein was still incomplete at the higher level of CaCl₂. The experiments of Huiskamp (3) with thymus nucleohistone showed that this desoxypentose nucleoprotein was insoluble in the presence of 0.9 per cent NaCl or 0.1 per cent CaCl₂. No experiments were conducted with mixtures of the two salts. No comparison is possible between the present results and those of Huiskamp (3) because in the latter case the precipitation of a desoxypentose nucleohistone was studied, while in the former the precipitation of a pentose nucleoprotein was involved.

Precipitation of Pentose Nucleoproteins from Rat Liver Extracts—The possibility that the precipitation of pentose nucleoproteins observed upon the addition of CaCl₂ to a rat liver homogenate (Fig. 1) might be due to an adsorption of the nucleoproteins on the larger formed elements of the cell was tested by removing the nuclei and mitochondria from the homogenate by centrifugation (6). In a preliminary experiment the resulting extract was treated with 0.055 per cent CaCl₂ on the basis of the data in Fig. 1. The results of these experiments are presented in Experiment 1, Table I. The data show that 1.31 mg. or 81 per cent of the PNA present in the liver extract was precipitated at this level of CaCl₂. These results are in contrast to the 1.97 mg. of PNA (93 per cent) precipitated from the whole liver

homogenate from which the extract had been prepared.   Thus although the precipitation of pentose nucleoprotein observed in the homogenate apparently did not involve adsorption upon the nuclei and mitochondria, the

TABLE I

Precipitation of Pentose Nucleoproteins from Rat Liver Homogenates Freed of Nuclei and Mitochondria

The results are expressed in terms of 4.0 ml. of solution containing 250 mg. of fresh rat liver or an equivalent amount of a nuclei and mitochondria free liver extract. Nucleoprotein was precipitated from 5.0 ml. of liver extract (equivalent to 167 mg. of fresh liver) by the addition of $CaCl_2$ in the concentrations indicated. The results in Experiments 1 and 2 are the average of two experiments, except as indicated. Pentose nucleic acid (PNA) and protein were determined in the whole homogenate and in the liver extract.   Protein was estimated in the supernatants from the $CaCl_2$ precipitates and PNA was measured in the precipitates and in the supernatants obtained after the addition of $CaCl_2$ as indicated.

Experi-ment No.		$CaCl_2$ concen-tration	PNA			Protein
			In homo-genate or extract	In $CaCl_2$ ppt.	In super-natant from $CaCl_2$ ppt.	
		per cent	*mg.*	*mg.*	*mg.*	*mg.*
1	Original liver homogenate	0	2.11			65.4
	$CaCl_2$ ppt. from homogenate	0.055		1.97		38.5*
	Nuclei and mitochondria free liver extract	0	1.61			47.0
	$CaCl_2$ ppt. from liver extract	0.055		1.31		16.1*
2	Nuclei and mitochondria free liver extract	0	1.67			49.3
	$CaCl_2$ ppt. from liver extract	0.017			1.56	1.2*
		0.025			1.46	1.5*
		0.033		0.75	1.01	5.8*
		0.041		1.12†	0.65†	9.1*†
		0.050		1.31	0.44†	12.6*
		0.066		1.44	0.28†	13.9*
		0.132		1.45	0.26†	
		0.198		1.42†	0.25†	

* These values were obtained by subtracting the protein found in the supernatant removed from the $CaCl_2$ precipitate from the protein found in the nuclei and mitochondria free liver extract or in the liver homogenate.

† Single values.

precipitation of nucleoprotein was considerably more effective in the homogenate than in the liver extract.   To determine whether any differences existed between the optimal $CaCl_2$ concentrations in the extract and the homogenate, the precipitation of PNA from the extract was studied at a

series of CaCl$_2$ concentrations. The results are presented in Experiment 2, Table I, and show that maximum precipitation of PNA was not reached in the case of the extract until the CaCl$_2$ concentration was 0.066 per cent. Precipitation of PNA was already maximum in the case of the homogenate at 0.033 per cent (Fig. 1, 0.4 ml. of CaCl$_2$). Even at the higher concentrations of CaCl$_2$, the precipitation of PNA was less complete in the extract (1.45 mg. or 86 per cent at 0.132 per cent CaCl$_2$). That the precipitation of PNA was indeed incomplete in the extract and not merely an experimental error was proved by measuring the PNA in the CaCl$_2$ precipitate as well as in the supernatant from the precipitate. For example, at 0.066 per cent CaCl$_2$, 1.44 mg. of PNA were found in the CaCl$_2$ precipitate and 0.28 mg. was found in the supernatant from the precipitate. Thus a total of 1.72 mg. of PNA was found in the two fractions. This compares favorably with the PNA present in the original extract (1.67 mg.).

Factors without Effect on Precipitation of Rat Liver Nucleoproteins by CaCl$_2$—The efficiency of the precipitation of nucleoproteins from rat liver homogenates by CaCl$_2$ was studied at tissue concentrations in the range of 31.2 to 125 mg. of fresh tissue per ml. No significant differences in the amounts of the nucleoproteins precipitated at the different tissue levels were observed. Since the concentration of the liver extracts used in the experiments described in the preceding section fell within the range of tissue concentration studied, it would appear that the tissue concentration was not a factor in the incomplete precipitation of nucleoproteins observed in the liver extract. Other factors which had no influence on the effectiveness of the precipitation of rat liver nucleoproteins by CaCl$_2$ were perfusion of the rat liver prior to homogenization and incubation of the homogenate at 0° for periods up to 24 hours prior to the addition of CaCl$_2$.

DISCUSSION

The experiments of Claude (2) demonstrated that a large portion of the acid-insoluble phosphorus of the liver cell was associated with particulate matter which could be sedimented from cell extracts by a centrifugal force of 18,000g. In our experiments, the addition of CaCl$_2$ to a rat liver homogenate produced virtually complete precipitation of the pentose nucleoproteins, and the question arises as to whether the CaCl$_2$ merely acted by agglutinating the particulate material of the liver cell sufficiently so that it could be sedimented at low speeds. The latter view was supported by experiments in which CaCl$_2$ precipitated a large part (85 per cent) of the pentose nucleoproteins of a liver extract containing only particulate material which would otherwise require high speed centrifugation for sedimentation.

The concentration of CaCl$_2$ which provided effective precipitation of the pentose nucleoproteins of liver homogenates approaches a physiological

level.   Thus the level of calcium ions (expressed as $CaCl_2$) in serum is about 0.027 per cent and the concentration of $CaCl_2$ which provided effective precipitation of the nucleoproteins from a rat liver homogenate was 0.033 per cent (Fig. 1).   These levels of $CaCl_2$ are considerably less than the 0.1 per cent $CaCl_2$ used by Huiskamp (3) to precipitate thymus nucleohistone and the 0.4 per cent $CaCl_2$ used by Brues, Tracy, and Cohn (1) to precipitate the nucleoproteins of rat liver.

The data clearly show (Fig. 1 and Table I) that a large part of the protein present in the liver homogenate or in the liver extract remains in solution after the addition of $CaCl_2$.   Whether $CaCl_2$ produces a precipitate which is entirely nucleoprotein in nature is very uncertain.   It is impossible to decide at present whether adsorption of soluble proteins occurs during the precipitation.[1]   On the other hand, if the action of calcium ions involves agglutination of particulate components of the cell, it would seem unlikely that the precipitate is pure, since there is no evidence to indicate whether these formed elements contain only one type of nucleoprotein or indeed whether the entire protein in the formed elements is associated with nucleic acids.

The experiments presented here are intended to serve as a model by means of which the precipitation of nucleoproteins by $CaCl_2$ and other inorganic salts can be studied in other tissues, since it is felt that the precipitation of nucleoproteins will prove of considerable use in problems such as fractionation of the soluble proteins of the cell and the purification of enzymes[1] in addition to a study of the nucleoproteins themselves.

SUMMARY

1. The precipitation of nucleoproteins from rat liver homogenates and from rat liver extracts by $CaCl_2$ was studied.

2. Maximum precipitation of the pentose nucleoproteins (93 per cent) present in rat liver homogenates was reached at a $CaCl_2$ concentration of 0.033 per cent.

3. Pentose nucleoproteins were incompletely precipitated by 0.033 per cent $CaCl_2$ from rat liver extracts which had been freed of nuclei and mitochondria.   Maximum precipitation of pentose nucleoproteins (85 per cent) from rat liver extracts was attained at a level of 0.066 per cent $CaCl_2$.   The pentose nucleic acid which had not been precipitated by the $CaCl_2$ was found in the supernatant from the $CaCl_2$ precipitate.

[1] Preliminary experiments in this laboratory (V. R. Potter, unpublished work) have indicated that as much as 30 to 50 per cent of the malic dehydrogenase of liver (a soluble protein) may be present in the $CaCl_2$ precipitate from liver homogenates. No data are as yet available to indicate the proportion of the soluble protein represented by the malic dehydrogenase or whether other soluble proteins behave similarly to the malic dehydrogenase in the $CaCl_2$ precipitation.

4. The desoxypentose nucleoproteins were completely precipitated at all levels of CaCl$_2$ presumably because these proteins were present in intact nuclei which were sedimented during the low speed centrifugation used in the experiments.

5. Sodium chloride was found to decrease the precipitability of the pentose nucleoproteins by CaCl$_2$ and the decreased precipitability was related to the NaCl concentration.

6. The precipitation of the liver pentose nucleoproteins by CaCl$_2$ was interpreted as an agglutination of particulate material in the liver suspensions.

BIBLIOGRAPHY

1. Brues, A. M., Tracy, M. M., and Cohn, W. E., *J. Biol. Chem.*, **155**, 619 (1944).
2. Claude, A., *J. Exp. Med.*, **84**, 61 (1946).
3. Huiskamp, W., *Z. physiol. Chem.*, **32**, 145 (1901).
4. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
5. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
6. Schneider, W. C., *J. Biol. Chem.*, **164**, 241 (1946).
7. Weichselbaum, T. E., *Am. J. Clin. Path.*, **16**, *Tech. Suppl.*, **10**, 40 (1946).

CHEMICAL AND ENZYMATIC PROPERTIES OF CRYSTALLINE CARBOXYPEPTIDASE

By FRANK W. PUTNAM* AND HANS NEURATH

WITH THE TECHNICAL ASSISTANCE OF ELAINE ELKINS AND S. SEGAL

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

Extensive investigation has been made of the physical, chemical, and enzymatic properties of the crystalline extracellular proteinases, pepsin, trypsin, and the chymotrypsins (1). However, despite the existence of the elegant specificity studies of Bergmann and coworkers (2–4), physical characterization by the newer methods of protein chemistry is lacking for carboxypeptidase, another crystalline proteolytic enzyme of the pancreas, first crystallized by Anson (5). Published physicochemical data on crystalline carboxypeptidase are limited to electrophoretic measurements in the Theorell apparatus, from which it was concluded that it migrates as an anion with constant velocity over the pH range 5.64 to 8.32 (6). No estimates of molecular weight or shape apparently have been made.

Since this peptidase is readily crystallizable (5) and has recently served as a useful tool in the structural analysis of biologically active peptides (6–9), it appeared desirable to undertake further investigation of its physicochemical properties by the methods of electrophoresis, diffusion, and viscosity.

It was also deemed of importance to reinvestigate the kinetics of hydrolysis of the historical substrate, chloroacetyl-l-tyrosine, by use of the highly purified enzyme. The enzymatic homogeneity of carboxypeptidase was disputed (10) until its specificity requirements toward a variety of substrates were elucidated by Bergmann and coworkers (3). However, as has been pointed out in a recent review (11), the literature contains few data on the kinetics of hydrolysis of chloroacetyl-l-tyrosine by the crystalline enzyme. Although Anson (12) has proposed a method for the estimation of the activity of the crystalline enzyme by use of chloroacetyl-l-tyrosine, the procedure involved the use of formaldehyde, later shown to have a strong inhibitory effect (3).

The present investigation is introductory to a general study of the characterization and mode of action of mammalian proteolytic enzymes of extracellular and intracellular origin.

* Present address, Loyola University School of Medicine, Chicago, Illinois.

Crystalline carboxypeptidase was prepared from sliced frozen beef pancreas by the method of Anson (5). Several modifications of this method were also investigated, including isoelectric precipitation by prolonged dialysis and fractional precipitation with ammonuim sulfate. The details of these methods will be described below in conjunction with electrophoretic analyses and measurements of the kinetics of the hydrolysis of specific substrates which were simultaneously performed as a guide for estimating the efficacy of the purification processes.

Kinetic Studies

Methods of Measurements—Two methods were employed in the estimation of the extent of hydrolysis of substrates: the submicro gasometric ninhydrin determination of free amino acids in the Van Slyke manometric apparatus (13), and the Grassmann and Heyde alcohol microtitration of the liberated amino groups (14).

Because the accuracy of the latter titration has been questioned (15), the method was first tested on solutions of pure amino acids. Experience indicated the desirability of conducting the titration in a nitrogen atmosphere to exclude absorption of carbon dioxide from the air. Accordingly, a side arm test tube was used as the titration vessel and a steady stream of nitrogen passed through an 18 gage stainless steel needle extending below the surface of the solution being titrated. The nitrogen served both to stir the liquid and to exclude carbon dioxide. A horizontal capillary burette of 1 cc. capacity, graduated to 0.01 cc., was employed.

With the aid of this modification, the results obtained for pure amino acids agreed to within ± 2 per cent with those given by the ninhydrin method and Kjeldahl nitrogen analysis.

In measurements of enzymatic hydrolysis, buffer composition and protein concentrations were chosen so as to minimize the turbidity resulting from precipitation on the addition of alcohol. Table I indicates the agreement found upon following the course of enzymatic hydrolysis of carbobenzoxyglycyl-*l*-phenylalanine[1] simultaneously by both the ninhydrin and titration methods.

Substrates containing a free carboxyl group were employed in the form of their sodium salts at a final concentration of 0.05 mM per cc., except that *dl* mixtures were made up to a final concentration of 0.10 mM per cc.

Tests indicated that enzyme action was stopped on the addition of absolute alcohol in the titration method and by the addition of the sample to

---

[1] We are indebted to Dr. A. A. Plentl, Cornell University Medical College, New York, for a generous gift of this compound.

boiling buffer, pH 2.5, in the ninhydrin method. Enzyme blanks and tests for the lability of the substrate were performed. Temperature of incubation was maintained by a water bath equipped with a suitable shaking device.

The buffer composition was 0.02 M phosphate-0.05 M LiCl, pH 7.65, in all cases unless otherwise stated. An exception is the hydrolysis of carbobenzoxyglycyl-l-phenylalanine at 25°, for which a 0.033 M phosphate buffer, pH 7.5, was employed to aid in comparison with the work of Hofmann and Bergmann (3). All pH values were measured with the glass electrode.

TABLE I

Hydrolysis of Carbobenzoxyglycyl-l-phenylalanine by Crystalline Carboxypeptidase at 25°

Time	Hydrolysis*		K = velocity constant†		C = proteolytic coefficient‡	
	Titration	Ninhydrin	Titration	Ninhydrin	Titration	Ninhydrin
min.	*per cent*	*per cent*	10^{-3} *min.*$^{-1}$	10^{-3} *min.*$^{-1}$		
10	10	7	4.8	3.3	12.3	8.5
25	22	21	4.2	4.0	10.9	10.4
60	39	42	3.6	4.0	9.2	10.3
90	48		3.1		8.1	
120	60	64	3.4	3.6	8.7	9.3
Average..					9.8	9.6

Carboxypeptidase, five times crystallized, pH 7.5, 0.033 M phosphate buffer.
* Estimated in 0.2 cc. samples.

$$† K = \frac{1}{\text{min.}} \log_{10} \frac{100}{100 - \% \text{ hydrolysis}}.$$

$$‡ C = \frac{K}{c} = \frac{K}{\text{mg. protein N per cc. test solution}} = \frac{K}{0.000388}.$$

Determination of Proteolytic Coefficients—Enzymatic activity was determined by following the kinetics of hydrolysis of several substrates. In keeping with accepted procedures (4) the results are expressed in terms of the proteolytic coefficient, C, defined as the first order reaction constant, K, divided by the enzyme concentration in mg. of protein N per cc. of test solution (K/c). To aid comparison with the crystalline carboxypeptidase used by Bergmann *et al.* (3, 4) carbobenzoxyglycyl-l-phenylalanine (CGlyP) was employed as the substrate of choice.[1]

The results given in Table I are comparable to those obtained by Bergmann *et al.* (3, 4), the proteolytic coefficient of about 9.6 being well within the range reported by these authors (8.6 to 12). No account was taken of possible inactivation of the enzyme upon storage in the cold.

In contrast to the kinetics of hydrolysis of CGlyP (Table I), the data of Tables II and III indicate that the hydrolysis of chloroacetyl derivatives does not follow the equation for a first order reaction (cf. also (3)). Nor do the data fit the equations for second order of reversible reactions. Since satisfactory rate constants were not obtained, it was not possible to calculate proteolytic coefficients in the usual manner.

However, it was found that by use of an empirical correction of the first order equation constants were obtained which enabled comparison of the

TABLE II

Hydrolysis of Chloroacetyl-l-tyrosine by Twice Crystallized Carboxypeptidase at 40° and pH 7.7*

Time	Hydrolysis	k	k'	Time	Hydrolysis	k	k'
Experiment 1. 7.11 × 10⁻³ mg. protein N per cc.				Experiment 2. 3.63 × 10⁻³ mg. protein N per cc.			
min.	*per cent*	10^{-3} *min.*$^{-1}$	10^{-3} *min.*$^{-1}$	*min.*	*per cent*	10^{-3} *min.*$^{-1}$	10^{-3} *min.*$^{-1}$
10.0	11	11.6	15.0	10	7	7.0	9.1
15.5	17	11.8	16.8	20	12	6.6	10.3
22.5	18	8.9	14.3	32	14	4.7	8.9
35.5	22	7.1	13.7	54	18	3.7	9.0
52.0	25	5.5	13.1	70	19	3.1	8.9
59.5†	28	5.4	13.7	95	21	2.5	8.8
63.0†	28	5.2	13.6	137	21	1.8	8.1
Experiment 3. 13.7 × 10⁻³ mg. protein N per cc.				Experiment 4. 1.83 × 10⁻³ mg. protein N per cc.			
6.5	15	25.2	29.7	16	7	4.7	6.9
13.0	24	21.3	28.6	27.5	8	3.1	5.4
18.0	28	18.0	26.3	51	11	2.2	5.3
25.0	35	17.3	27.9	92	12	1.4	5.1
32.5†‡				145‡			

$$k = \frac{2.3}{t} \log_{10} \frac{100}{100 - \% \text{ hydrolysis}}; k' = k + k_2 x.$$

* Measured in 0.2 cc. samples by alcohol titration.

† Tyrosine crystallization.

‡ No additional hydrolysis observed.

rates of hydrolysis of both chloroacetyl-*l*-tyrosine (ClAcT) and chloroacetyl-*l*-phenylalanine (ClAcP) under different conditions of enzyme concentration and activity. The equation used was $k' = k + k_2 x$, where k is the specific reaction rate calculated from the first order equation,[2] k_2 is the empirical correction factor, equal to 30×10^{-3}, and x is the fraction

[2] It is to be noted that natural logarithms were used for these calculations (Tables II and III), in accordance with theory, whereas, in calculations of rate constants (K) of the hydrolysis of carbobenzoxy derivatives, $\log_{10}$ values were used. This was done to aid in the comparison with the values of Bergmann et al. (3, 4).

hydrolyzed. Typical data are presented in Tables II and III for measurements at 40° and 28°. All samples of purified carboxypeptidase were found to obey the above equation. Inconclusive results were obtained with the crude pancreatic exudate.

In the 4-fold range of concentration (3.6 to 13.7 × 10⁻³ mg. of protein N per cc.) which was found convenient both with regard to time and degree of hydrolysis of ClAcT by twice crystallized carboxypeptidase, k' was approximately proportional to enzyme concentration. Hence, the activity of the enzyme toward this substrate, as well as toward ClAcP (cf. Table III), could be indicated by modified proteolytic coefficients,[2] k'/c.

TABLE III

Hydrolysis* of Chloroacetyl-l-phenylalanine by Seven Times Crystallized Carboxypeptidase† at 28° and pH 7.7

Time	Hydrolysis	k	k'	Time	Hydrolysis	k	k'
Experiment 1. 3.9 × 10⁻³ mg. protein N per cc.				Experiment 2. 11.4 × 10⁻³ mg. protein N per cc.			
min.	*per cent*	10^{-3} *min.*⁻¹	10^{-3} *min.*⁻¹	*min.*	*per cent*	10^{-3} *min.*⁻¹	10^{-3} *min.*⁻¹
10	12	13.2	16.9	5	15	37.5	41.9
20	20	11.1	17.1	10	24	27.3	34.5
30	19	7.0	12.7	20	37	23.0	34.1
45	28	7.2	15.5	30	47	21.2	35.2
60	37	7.6	18.6	45	54	17.4	33.7
90	37	5.1	16.2	60	69	19.5	40.2
120	40	4.2	16.2				

* Measured in 0.2 cc. samples by the ninhydrin method.

† K/c on CGlyP = 10.3; k'/c on ClAcT = 3.1. This enzyme preparation had been stored in the ice box for 2 months.

Purification of Carboxypeptidase

Crystalline carboxypeptidase was prepared from sliced frozen beef pancreas by the method of Anson (5). Recrystallization by this method involves prolonged suspension of the crystals in dilute alkali. While a highly active product was obtained, considerable loss of material was incurred, owing to the limited solubility of the crystals, even at pH 10. The form of crystals obtained after five crystallizations by this method is shown in Fig. 1 (upper slide).

Alternative procedures in which exposure to alkali was avoided were explored and it was found that recrystallization could be achieved by dissolving the crystals in 5 per cent LiCl and dialyzing the solution against distilled water in the cold. The habit of these crystals sometimes differed from that of those obtained by Anson's method, the former being of the large tabular type, as shown in the center slide of Fig. 1. This procedure is

more successful when applied to material previously crystallized rather than to the crude euglobulin precipitate.

Fractional precipitation of the proteins of the pancreatic exudate with ammonium sulfate was also explored. The fraction precipitated in the cold by 0.4 saturated ammonium sulfate at pH 5.8 contained one-third of the total protein and had a proteolytic coefficient toward ClAcT of 1.3 as compared to about 0.7 for the whole exudate. However, two additional precipitations under the same conditions neither yielded a crystalline prep-
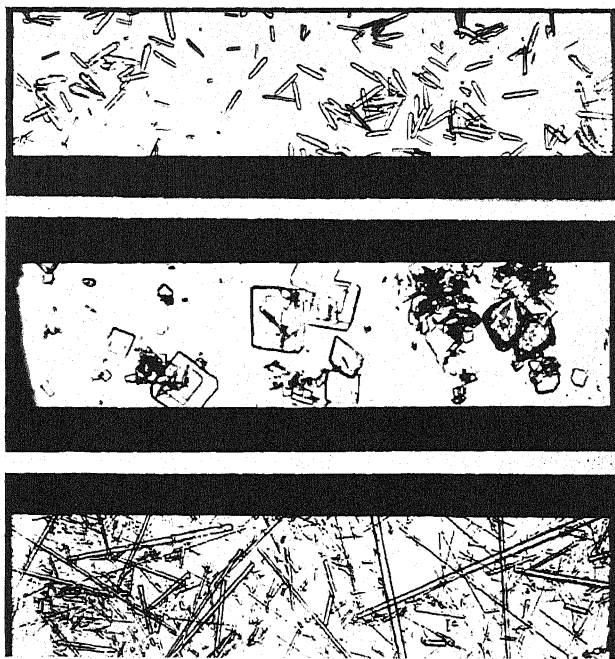
FIG. 1. Photomicrographs of crystalline forms of carboxypeptidase. Upper slide, five times crystallized by Anson's method; middle slide, the same, recrystallized by prolonged dialysis; lower slide, crystals having half the maximum activity of pure carboxypeptidase, obtained by salt precipitation followed by dialysis against 2 per cent NaCl. All enlargements 100 ×.

aration nor increased enzymatic activity. Since an aliquot of the same exudate readily yielded crystalline carboxypeptidase when purified by Anson's method, it was concluded that fractional precipitation with ammonium sulfate was not suitable.

However, when a suspension of the precipitate obtained after three precipitations by 0.4 saturated ammonium sulfate was adjusted to pH 5.0 and dialyzed in the cold against 2 per cent NaCl, a heavy crystalline precipitate, consisting of long prisms, appeared after standing for 1 week

(lower slide, Fig. 1). The crystals were essentially insoluble in distilled water but could readily be dissolved in the buffer used for activity measurements. Qualitatively, this protein was enzymatically active toward ClAcT and toward CGlyP, the proteolytic coefficient toward the latter substrate being about 5. The unusual behavior of this crystalline material in electrophoresis is described below.

Electrophoretic and Enzymatic Analayses—The progress of purification was followed by electrophoresis and by determination of the proteolytic coefficients toward ClAcT and CGlyP. Electrophoresis was carried out in

TABLE IV

Electrophoretic Study of Progress of Purification of Carboxypeptidase

Experiment No.	Material	pH*	Mobility,[†] 10^{-5} sq. cm. volt^{-1} sec.$^{-1}$				Per cent area[‡]			
			Component 1	Component 2	Component 3	Component 4	Component 1	Component 2	Component 3	Component 4
1	Pancreatic exudate	7.65	?	−2.06	−5.42	−13.1	14.8	28.4	24.4	32.4
2	Euglobulin ppt.	7.68	?	−2.10	−5.38	−13.1	10.7	46.6	14.7	28.0
3	3 times crystallized	7.68	−1.4	−2.06			(15)	85		
4	5 " "	7.62	−1.5	−2.10			Insufficient separation			
5	7 " "	8.50		−1.76	−4.86				93.7	(6.3)§
6	Crystals from salt pptn.	7.70		−1.90	−5.29		Anomalous distribution			
7	Crystals from salt pptn.	8.50		−1.67	−4.88		Anomalous distribution			

The figures in parentheses indicate approximate values.

* Buffer composition, 0.04 M phosphate-0.1 N LiCl, ionic strength approximately 0.2, except for Experiments 5 and 7 which were done at pH 8.50, in 0.1 N Na veronal-0.1 N LiCl buffer, ionic strength of 0.2.

† Calculated from the descending pattern.

‡ Measured from the ascending pattern for Experiments 1 and 2 because of better resolution; all others from the descending pattern.

§ Two section cell used; no separation observed on ascending side.

the Tiselius apparatus, as previously described (16); further experimental details are given in Tables IV and V. The electrophoretic patterns obtained in Experiments 1 to 12 (Tables IV and V) are shown in Fig. 2.

The clarified pancreatic exudate contains four electrophoretic components,[3] designated in Table IV in order of increasing mobility at pH 7.65 as Components 1, 2, 3, and 4. It is interesting to note that this exudate contained about 1 per cent protein, of which some 28 per cent consisted of Component 2, which was subsequently identified as carboxypeptidase on the basis of mobility and activity. Relatively little purification was ac-

[3] On the descending (right-hand) side, Component 1 was not resolved.

complished by euglobulin precipitation (compare Experiments 1 and 2), though it enabled concentration of the protein. However, crystallization is accompanied by the removal of the faster moving Components 3 and 4 (Experiment 3), although the skewness of the boundary of Component 2, remaining even after five crystallizations (Experiment 4), is indicative of the presence of a small proportion of the slowest moving component. The material obtained after seven crystallizations when studied at pH 8.5 appears to be electrophoretically homogeneous (Experiment 5) except for a

TABLE V

Electrophoretic Mobility* at 1° of Crystalline Carboxypeptidase in Monovalent Buffer Solutions of 0.2 Ionic Strength

Experiment No.	pH†	Buffer‡	Time	Field strength	Mobility§	Remarks
			sec.	*volts per cm.*	10^{-5} *sq. cm.* $volt^{-1} sec.^{-1}$	
8	9.30	0.15 N NaOH-0.1 N glycine-0.185 N LiCl	14,400	2.50	−1.69	Increased fast component
9	8.51	0.02 N HV-0.1 N NaV-0.1 N LiCl	14,400	2.86	−1.80	Fast component comprises 6%
10	6.60	0.004 N HCac-0.02 N NaCac-0.18 N LiCl	28,800	2.56	−0.52	Apparently homogeneous
11‖	5.95	0.02 N HCac-0.02 N NaCac-0.18 N LiCl	14,400 25,800	2.61	0	Isoelectric point
12	5.40	0.02 N NaAc-0.18 N LiCl	25,200	2.43	+0.52	Apparently homogeneous

* Data calculated from the descending pattern only.

† All pH values were determined at 25°. No correction was made to 0° except for the glycine-sodium hydroxide buffer (pH 8.80 at 25°), the pH value of which was determined from the data of Sørensen (17) extrapolated to 1°.

‡ V denotes diethyl barbiturate, Cac, cacodylate, Ac, acetate.

§ Major component only.

‖ Lower boundary 14,400 seconds, upper boundary 25,800 seconds (see Fig. 2).

trace of a faster moving component observable only on the descending side. No attempt has yet been made to identify the nature of the other electrophoretic components seen in the pancreatic exudate.

In analogy with electrophoretic data, determinations of protelytic coefficients reveal little purification when the euglobulin precipitate is compared to the clarified pancreatic exudate (Table VI). However, crystallization achieves a significant increase in activity, maximum values of the proteolytic coefficients for both substrates, ClAcT and CGlyP, being ob-

tained after five crystallizations.   Qualitative correlation between the increase in electrophoretic homogeneity upon purification and increase in the proteolytic coefficient toward ClAcT is observed.
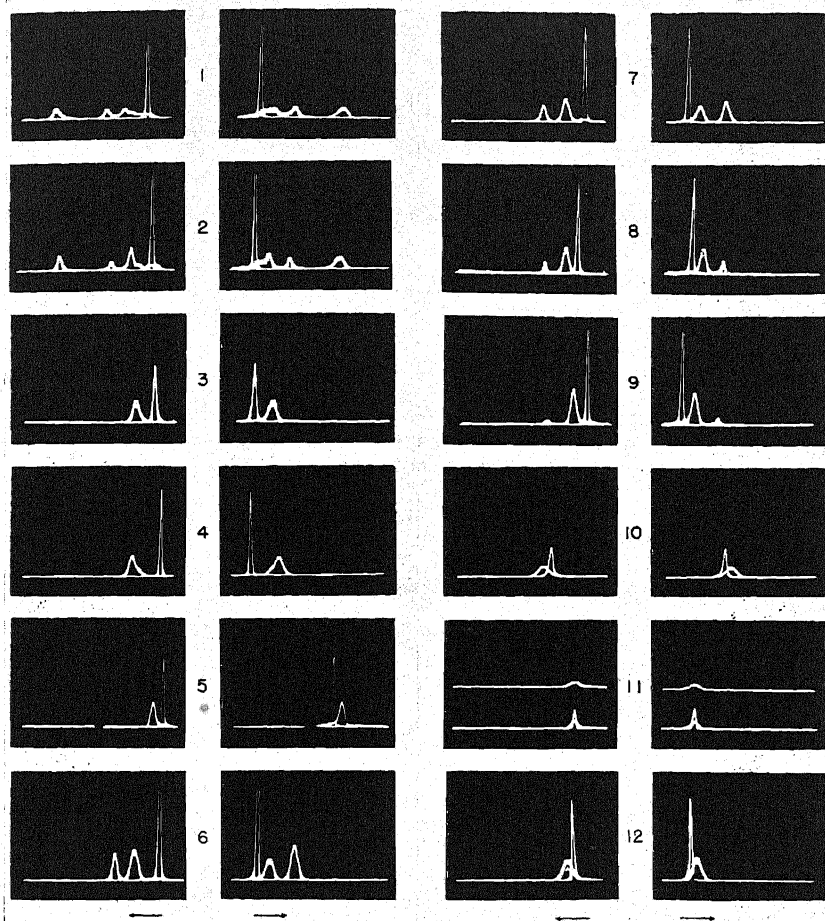
FIG. 2. Electrophoretic patterns of crude and crystallized carboxypeptidase preparations.   For the conditions of Experiments 1 to 7 see Table IV, and of Experiments 8 to 12, Table V.   The arrows denote the direction of migration, right, descending; left, ascending boundary.   The sharp initial boundary in all cases indicates the starting point.

The electrophoretic properties of solutions of the crystals obtained after fractional precipitation with ammonium sulfate (shown in Fig. 1, lower slide), are given in Experiments 6 and 7 (Table IV and Fig. 2).   At both pH values studied, two distinct components were observed.   Inspection

reveals that the ascending and descending patterns are anomalous with respect both to component distribution and distance of migration from the origin, a phenomenon indicative of component interaction (18). Nevertheless, the slower moving boundary on the descending side may be identical with carboxypeptidase, since its mobility at two different pH values (7.7 and 8.5) agrees with that of the major component of the several times crystallized enzyme (compare Experiments 4 and 6 and Experiments 5 and 7). It is probable that the faster component in Experiment 6 is identical with Component 3 of the clarified exudate and the residual component in the seven times crystallized material (Experiment 5).

TABLE VI

Change in Enzymatic Activity on Purification of Carboxypeptidase

Material	Proteolytic coefficients	
	$\dfrac{k'}{c}$ (ClAcT)*	$\dfrac{K}{c}$ (CGlyP)†
Pancreatic exudate....................	0.7	
Euglobulin ppt........................	0.9	
Once crystallized......................	1.8	
Twice "	2.1	
3 times crystallized...................	2.9	
4 " "	3.8	
5 " "	3.8	9.8
7 " "	3.1	10.3
8 " "		11.1

For definition of k'/c and K/c see the text.

* Titration method, incubation at 40°, pH 7.7.

† Ninhydrin method, incubation at 25°, pH 7.5.

Physicochemical Properties of Purified Carboxypeptidase

Electrophoretic Homogeneity, Mobility, and Isoelectric Point—The electrophoretic mobility was determined in monovalent buffers of 0.2 ionic strength over the pH range 9.3 to 5.4. Inspection of the data (Experiments 8 to 12, Table V and Fig. 2) reveals a single component of very low mobility below pH 8.5, whereas, at higher pH ranges, a minor, faster moving component is apparent. At pH 9.3, the proportion of faster moving component is 20 per cent of the seven times crystallized enzyme preparation (Experiment 8), while the same preparation at pH 8.5 (Experiment 5, Table IV and Fig. 2) reveals only 6 per cent of a fast component (on the descending side only). In the five times crystallized material at pH 8.5 (Experiment 9) the fast moving component is apparent on both sides and likewise comprises 6 per cent.

The results of electrophoretic mobility measurements over the pH range studied are plotted in Fig. 3. The shape of the pH-mobility curve is similar to that of other globulins, the enzyme being isoelectric near pH 6.0. This is further confirmed by the negligible mobility observable[4] in Experiment 11. In monovalent buffers, the enzyme seems to be least soluble at pH 6, as witnessed by the diminished area of the electrophoretic pattern (Fig. 2).[4] The slope of the pH-mobility curve at the isoelectric point, $\delta U/\delta pH$, is 0.86 $\times$ 10^{-5}. The activity of the enzyme solutions removed from the Tiselius cell, when measured on CGlyP at pH 7.5, was found to decrease with decreasing pH. Though it has been reported that crystalline carboxypepti-
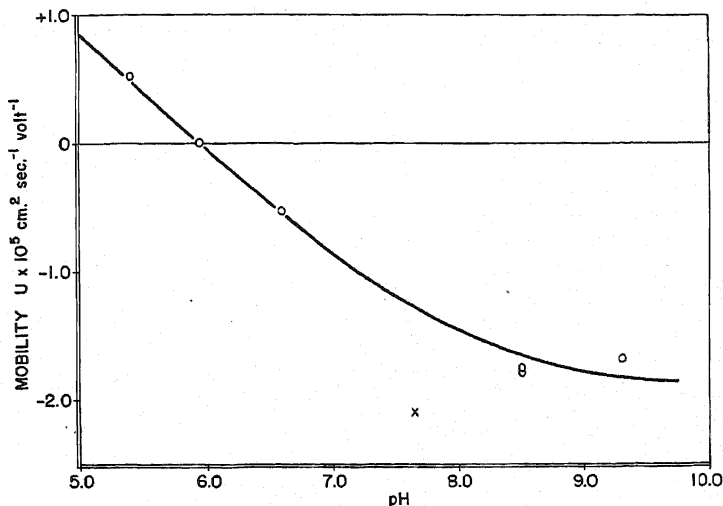
FIG. 3. The electrophoretic mobility-pH curve of crystalline carboxypeptidase in monovalent buffers of 0.2 ionic strength. $\times$ indicates the mobility of several preparations of carboxypeptidase in polyvalent buffer used for activity measurements (ionic strength, 0.2).

dase is inactivated at pH 5.0 and below (5, 6), about 40 per cent of the maximum activity toward CGlyP was still observable at pH 5.4.

Diffusion and Viscosity—Diffusion measurements were carried out at 25° with the refractometric scale apparatus and technique already described (19). The results of measurements made at two different enzyme concentrations are given in Table VII. Agreement among the values obtained by different methods of calculation was as expected for a monodisperse solution. The maximum ordinate calculated from the normalized curve deviated less than 1 per cent from the ideal value. Also, no shift could be

[4] In these experiments the enzyme was dissolved at maximum concentrations obtainable at 1° in the buffers used. Electrolysis was extended over a 7 hour period.

<div align="center">

TABLE VII

Diffusion Constant* of Crystalline Carboxypeptidase

</div>

	Time	D_1	D_2	D_3	D_4	D_5
	sec.	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}
8 times crystallized, protein concentration 0.7 %†	20,760	(10.07)	(10.21)	(10.35)	(10.51)	
	38,280	9.59	9.58	9.55	9.73	9.01 ± 0.1
	95,880	9.56	9.46	9.34	9.42	9.25 ± 0.2
Average......................		9.45 ± 0.21 × 10^{-7} sq. cm. sec.$^{-1}$				
D'...........................		9.95 × 10^{-7} sq. cm. sec.$^{-1}$				
7 times crystallized, protein concentration 0.43 %‡	37,800	9.69	9.67	9.63		9.41 ± 0.4
	78,300	9.58	9.35	9.12		
Average......................		9.49 ± 0.21 × 10^7 sq. cm. sec.$^{-1}$				
D'...........................		9.94 × 10^{-7} sq. cm. sec.$^{-1}$				

The figures in parentheses indicate early time values not considered in averaging the data (19, 20).

* D denotes the diffusion constant at 25° in sq. cm. per second; D_1, D_2, D_3, D_4, and D_5 refer, respectively, to the diffusion constant calculated by the maximum height, maximum height-area (unsquared), maximum height-area (squared), standard deviation, and successive analysis methods (19, 20). D' is the average diffusion constant corrected for solvent viscosity.

† Buffer composition, 0.04 M phosphate-0.4 N NaCl, pH 7.5.

‡ Buffer composition, 0.04 M phosphate-0.1 N LiCl, pH 7.65.
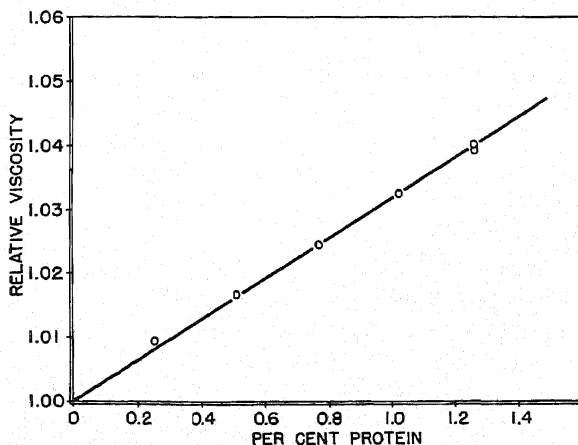
FIG. 4. Relative viscosity of eight times crystallized carboxypeptidase plotted against the protein concentration in weight per cent.

detected in values obtained along the curve by the method of successive analysis. These criteria indicate molecular homogeneity within the limits of the resolution of the method (19).

Viscosities were measured in capillary viscometers at 25°, as already described (21). The solvent was a 0.04 M phosphate-0.4 N NaCl buffer, pH 7.5. The linear relation of relative viscosity to protein concentration (in weight per cent) is shown in Fig. 4.

TABLE VIII

Hydrolysis of Synthetic Substrates by Crystalline Carboxypeptidase

Substrate*	Carboxy-peptidase†	pH	Temperature	Time	Hydrolysis	$\frac{K}{c}$	$\frac{k'}{c}$
	mg. N per cc.		°C.	min.	per cent		
Carbobenzoxyglycyl - l - phenylalanine (see Table I)	0.00039	7.5	25	25	21	9.6	
				60	39		
Chloroacetyl - l - tyrosine (see also Table II for twice crystallized enzyme preparation)	0.0039	7.7	40	10	10		3.8
				20	13		
				30	15		
Chloroacetyl - l - phenyl-alanine (see Table III)	0.0039	7.7	28	10	12		4.2
				20	20		
				60	37		
Acetyl - dl - tryptophane	0.083	7.7	37	16	8.5		
				23	10.4		
				45	13.5		
				72	14.4		
				105	17.9		
Formyl-l-phenylalanine	0.119	7.5	25	31	20		
				60	33.7		
				120	51.6		
				hrs.			
Carbobenzoxyglycyl-glycine§	0.119	7.5	25	12	9.2	0.00035	
				24	12.1		
				48	24.0		
				72	36.8		
Benzoyl-l-argininamide	0.083	7.7	40	30	0		
Acetylglycine	0.229	7.7	37	24	0		
dl-Leucylglycine	0.229	7.7	37	42	0		

* Hydrolysis was followed by the ninhydrin method except for measurements of chloroacetyl-l-tyrosine, acetyl-dl-tryptophane, and benzoyl-l-argininamide, for which the titration method was used. Substrate concentration 0.05 M in all cases. Unless otherwise indicated in this paper, the substrates were either prepared in this laboratory or received from members of the Department.

† Five or seven times recrystallized.

§ Received through the courtesy of Dr. J. S. Fruton.

Molecular Constants—Apparent molecular shapes, hydration being neglected, b/a, and assuming 30 per cent hydration, $(b/a)h$, were calculated from the limiting slope of the viscosity curve by graphical solution of the Simha equation (21). The values obtained, assuming prolate ellipsoids, were b/a 3.6, and $(b/a)h$ 2.1 where b/a is the ratio of major to minor axis.

The dissymmetry constant of the unhydrated molecule, f/f_0, was accordingly 1.16. These data indicate that, in comparison to many other proteins, carboxypeptidase has a remarkably low degree of molecular dissymmetry.

The molecular weight calculated from the dissymmetry constant, from the diffusion constant, and an assumed partial specific volume of 0.75 (19), is 31,600.

Enzymatic Specificity of Purified Carboxypeptidase

The objects of these kinetic measurements were to determine the specificity of the physically characterized enzyme preparation toward established substrates for this enzyme as well as toward other substrates not previously tested.   Tests on typical substrates for other extracellular proteolytic enzymes (e.g., benzoyl-l-argininamide[5] for trypsin, and dl-leucylglycine for dipeptidase (4, 22)) were also made as a check for the enzymatic purity of the present preparations.   Representative results are given in Table VIII.   The results for the rate of hydrolysis of CGlyP, ClAcT, and ClAcP have already been given in detail and are presented in brief in Table VIII merely to aid in the comparison with those of the other substrates included in this table.   It should be noted that the value of $k'/c = 3.8$ for the hydrolysis of ClAcT by five times crystallized carboxypeptidase represents the increased activity of the enzyme upon purification, for twice crystallized carboxypeptidase yielded a value of only 2.0 to 2.45 (cf. Table II).

DISCUSSION

The electrophoretic behavior of carboxypeptidase is consonant with its properties of a typical euglobulin.   The low slope of the pH-mobility curve in the region near the isoelectric point is consistent with the limited solubility of the enzyme in dilute salt solutions, and the observed isoelectric point, pH 6.0, is within the range of precipitation of the enzyme in salt-free solutions (pH 4.5 to 7).   The mobility data obtained in this work with the Tiselius electrophoresis apparatus differ from early results obtained with the Theorell apparatus (6).   Likewise, the isoelectric point determined from the pH-mobility curve differs from the value, pH 4.4, obtained by the microelectrophoretic method on a supension of crystals (23).

Isoelectric precipitation, either by pH adjustment of a suspension of crystals, proposed by Anson (5), or by dialysis of a neutral salt solution of the enzyme, proved suitable for purification, different forms of crystals being obtained, however.   On the other hand, fractional precipitation of the proteins of the pancreatic exudate with ammonium sulfate failed to

---

[5] Obtained from Dr. J. P. Greenstein, National Cancer Institute, Bethesda, Maryland.

yield satisfactory purification of the enzyme. One crystalline fraction obtained after salt fractionation exhibited only one-half of the maximum enzymatic activity of purified carboxypeptidase and contained a large amount of another electrophoretic component.

No attempt has yet been made to identify the other electrophoretic components of pancreatic exudate. Presumably, they correspond to the several enzymatic constituents of pancreatic juice, i.e. trypsin, chymotrypsin, etc., or their respective inactive precursors. It is of interest that in a recent study Munro and Thomas (24) reported the presence of four to six enzymatically unidentified electrophoretic components in stimulated canine pancreatic juice, the number depending on the buffer used and on individual variations.

Despite the apparent lack of electrophoretic homogeneity at certain pH values, crystalline carboxypeptidase appears to be molecularly homogeneous as shown by an analysis of the diffusion curves. However, since the reported molecular weights of other proteolytic enzymes of pancreatic origin are of the same magnitude (1), this may not be a critical test. The invalidity of crystallinity as a criterion of enzyme purity is indicated both by increasing activity on repeated crystallization and by electrophoretic inhomogeneity.[6]

First order kinetics for the hydrolysis of peptide derivatives by carboxypeptidase have not been found in this work or reported in the more extended studies of Bergmann and coauthors (2-4) for other than carbobenzoxy derivatives. However, empirical corrections of the first order specific reaction rates of the hydrolysis of ClAcT and ClAcP enable the calculation of modified proteolytic coefficients for these substrates which are approximately proportional to enzyme concentration. Although it remains to be unequivocally established that inhibition is exclusively due to the chloroacetate ion, suggestive evidence is provided by (1) the dependence of the degree of inhibition on the amount of substrate hydrolyzed, at all enzyme concentrations tested, and (2) the identical correction factors, $k_2 = 30 \times 10^{-4}$, for both chloroacetyl derivatives. Moreover, the data of Hofmann and Bergmann (3) for the inhibition of the hydrolysis of carbobenzoxyglycyl-l-phenylanaline by chloroacetate fit the present empirical equation when recalculated on a common numerical basis.[2] Since the same correction factor applies when rates of hydrolysis are measured at 37° and 28°, a contributory influence of enzyme inactivation on in-

---

[6] Although the electrophoretic impurity observed in the alkaline pH range may be ascribed to the result of alkali denaturation, it should be remembered that crystalline horse serum albumin shows a similar behavior; i.e., is electrophoretically homogeneous over a wide pH range but gives anomalous boundaries in another region which, however, is well within the pH stability range of the protein (25).

hibition may be discounted. It is noteworthy that within the errors inherent in the use of the modified proteolytic coefficient, k'/c, the values for ClAcP and ClAcT are the same. This is in agreement with the findings of Bergmann and Fruton (4) who have shown that for a series of carbobenzoxy derivatives of phenylalanine- and tryrosine-containing substrates the quotient of the proteolytic coefficients was constant; *i.e.*, 1.6 to 1.8.

Neither the hydrolysis of acetyl-*dl*-tryptophane nor of formyl-*l*-phenylalanine follows a first order law. The proteolytic coefficient, K/c, toward carbobenzoxyglycylglycine[7] obtained in this work is comparable to that calculated from the data of Hofmann and Bergmann (3); *i.e.*, 3.5×10^{-4} as compared to 2.1×10^{-4}. No activity was found toward benzoyl-*l*-argininamide, *dl*-leucylglycine (4, 22), or acetylglycine.

SUMMARY

The preparation of crystalline carboxypeptidase from bovine pancreas by the methods of isoelectric precipitation and fractional precipitation with ammonium sulfate has been studied, and the course of purification followed by electrophoretic and enzymatic analysis. After repeated crystallization, the enzyme showed nearly constant activity toward several substrates, was apparently molecularly homogeneous in diffusion, and apparently homogeneous in electrophoresis between pH 5.4 and 6.6. However, at pH 8.5 and higher a minor component appeared, the nature of which is undetermined.

The molecular constants of eight times crystallized carboxypeptidase have been determined by the methods of viscosity, diffusion, and electrophoresis. The molecular weight is about 32,000, the enzyme molecule having a low degree of dissymmetry. The isoelectric point determined from the pH-mobility curve was about pH 6.0.

The activity of the purified enzyme toward a number of substrates has been investigated. The kinetics of the hydrolysis of chloroacetyl-*l*-tyrosine and chloroacetyl-*l*-phenylalanine have been studied, and an empirical equation enabling the calculation of proteolytic coefficients has been presented.

BIBLIOGRAPHY

1. Northrop, J. H., Crystalline enzymes, New York (1939).
2. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117**, 189 (1937).
3. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134**, 225 (1940).

[7] Obtained from Dr. J. S. Fruton, Yale University, New Haven.

4. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **145,** 247 (1942).

5. Anson, M. L., *J. Gen. Physiol.*, **20,** 663 (1937).

6. Ågren, G., and Hammarsten, E., *J. Physiol.*, **90,** 330 (1937).

7. Plentl, A. A., and Page, I. H., *J. Exp. Med.*, **79,** 205 (1944).

8. Plentl, A. A., and Page, I. H., *J. Biol. Chem.*, **163,** 49 (1946).

9. Croxatto, H., Croxatto, R., and Rioseco, M., *Rev. Soc. argent. biol.*, **21,** 23 (1945).

10. Abderhalden, E., and Abderhalden, R., *Fermentforschung*, **16,** 48 (1938).

11. Johnson, M. J., and Berger, J., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2,** 69 (1942).

12. Anson, M. L., *J. Gen. Physiol*, **20,** 781 (1937).

13. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141,** 627 (1941).

14. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183,** 32 (1929).

15. Plentl, A. A., and Page, I. H., *J. Biol. Chem.*, **155,** 363 (1944).

16. Sharp, D. G., Cooper, G. R., and Neurath, H., *J. Biol. Chem.*, **142,** 203 (1942).

17. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 206 (1928).

18. Longsworth, L. G., and MacInnes, D. A., *J. Gen. Physiol.*, **25,** 507 (1942).

19. Neurath, H., *Chem. Rev.*, **30,** 357 (1942).

20. Putnam, F. W., Erickson, J. O., Volkin, E., and Neurath, H., *J. Gen., Physiol.*, **26,** 513 (1943).

21. Neurath, H., Cooper, G. R., and Erickson, J. O., *J. Biol. Chem.*, **138,** 411 (1941).

22. Greenstein, J. P., *J. Biol. Chem.*, **112,** 517 (1935–36).

23. Anson, M. L., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Lepizig, **7,** 118 (1938).

24. Munro, M. P., and Thomas, J. E., *Am. J. Physiol.*, **145,** 140 (1945).

25. Sharp, D. G., Cooper, G. R., Erickson, J. O., and Neurath, H., *J. Biol., Chem.*, **144,** 139 (1942).

THE BIOLOGICAL UTILIZATION OF GLYCINE FOR THE SYNTHESIS OF THE PROTOPORPHYRIN OF HEMOGLOBIN

By DAVID SHEMIN AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

Structural considerations and experimental findings (1–3) suggest that at least two organic precursors are involved in the biological synthesis of the protoporphyrin of hemoglobin. An obvious nitrogenous precursor may be an amino acid but this need not be one having a cyclic structure. In fact, from theoretical considerations there are many objections to the postulation of biological incorporation of some preformed ring into the porphyrin structure.

Evidence has been obtained in this laboratory that the nitrogenous precursor of the protoporphyrin of hemoglobin of the human erythrocyte is glycine (1, 2). As a further study of this subject in man would be expensive, we have used the rat for an investigation of the utilization of isotopic glycine, glutamic acid, proline, leucine, and ammonia for the synthesis of heme. Proline and glutamic acid were selected since it has often been suggested, because of the similarity of their structures to that of the pyrroles, that proline and the anhydride of glutamic acid, pyrrolidonecarboxylic acid, may be precursors of the protoporphyrins (4). Leucine was chosen as a representative α-amino acid whose intact carbon chain is unlikely to be used for pyrrole synthesis. Ammonia was chosen in order to test the non-specific utilization of nitrogen liberated by deamination of amino acids.

In order to compare the utilization of these compounds for porphyrin synthesis, they were labeled with isotopic nitrogen and fed individually to rats on a protein-free diet. Glycine and ammonia, as ammonium citrate, were fed to rats at a level of 1.33 mM per 100 gm. of body weight per day over a period of 3 days. In the case of the racemic amino acids twice the amount was administered over a period of 3 days, 2.66 mM per 100 gm. of body weight per day. The animals were given a protein-free diet in order to avoid dilutions of varying magnitudes of the test substance which would be caused by amino acids of dietary proteins. The hemin was isolated 2 weeks after the feeding of the isotopic test substances, for it had previously been found in a human experiment that at this time the N^{15} concentration of the porphyrin was near to its maximum value (2). The isotope concentrations in the compounds fed and in the hemin isolated are given in Columns 2 and 3 of Table I.

Since the N^{15} concentrations of the test compounds were different, the isotopic values obtained in the hemin preparations were calculated on the basis that the compound fed contained 100 per cent N^{15}. This is done to facilitate the comparison of the utilization of the different compounds for porphyrin formation (see Column 4, Table I). These results clearly demonstrate that glycine is by far the most effective source of nitrogen for the synthesis of heme. The values given for *dl*-leucine and *dl*-proline are not actually on a comparable basis to those for glycine or ammonia. In the case of these *dl*-amino acids an equal amount of the labeled *d* isomer was given along with the *l* isomer. It is known that a large part of the nitrogen

TABLE I

Comparison of N^{15} Concentration in Hemin after Feeding Isotopic Compounds

Compound fed		Hemin N^{15} concentration	Hemin N^{15} concentration*
Compound (1)	N^{15} concentration (2)	(3)	(4)
	atom per cent excess	*atom per cent excess*	*atom per cent excess*
Glycine	11.6	0.108	0.93
"	19.0	0.169	0.89
Ammonium citrate	13.0	0.012	0.09
dl-Glutamic acid	18.6	0.032	0.17
dl-Proline	11.6	0.031	0.27† (0.18)
"	11.6	0.028	0.24† (0.15)
dl-Leucine	32.7	0.051	0.16† (0.07)

* Calculated on the basis that the compound fed contained 100 per cent N^{15},

$$\frac{N^{15} \text{ concentration in hemin (Column 3)}}{N^{15} \text{ concentration of compound fed (Column 2)}} \times 100 = \text{Column 4}$$

† To correct for the *d* isomers 0.09 per cent should be subtracted from these values. Corrected values would be 0.18, 0.15, and 0.07 per cent respectively. See the text for explanation.

of these unnatural isomers is liberated, probably in the form of ammonia. In these cases we were thus feeding not only the labeled *l*-amino acid but also, in potential form, an equivalent amount of labeled ammonia. To bring the values of these compounds to a comparable basis with ammonia and glycine one should subtract 0.09 atom per cent excess N^{15}, the isotope concentration found in the hemin after the feeding of ammonia, from the values given in Column 4. The corrected isotope concentration in the hemin is therefore only 0.18 and 0.15 per cent for the two proline feedings and 0.07 per cent for the leucine feeding. These considerations do not apply to the experiment in which *dl*-glutamic acid was administered, for it is known that *d*-glutamic acid is not metabolized, but largely excreted (5). The isotope concentrations found in the porphyrin after the feeding of ammonia, glutamic acid, proline, and leucine are therefore not significantly

different from each other and about one-thirteenth to one-fifth of that found after the feeding of glycine. The isotope concentration found in the hemin after the feeding of these compounds represents values one would expect to find after the feeding of a non-specific source of isotopic nitrogen which would only enrich the N^{15} concentration of the body nitrogen from which the precursor of heme is synthesized. The slightly higher values found after the feeding of proline and glutamic acid may be the result of a more direct utilization of the l isomers of these two acids for the formation of glycine than of leucine or ammonia. An indication of this difference has been obtained (6).

Enol of β-ketoaldehyde + glycine
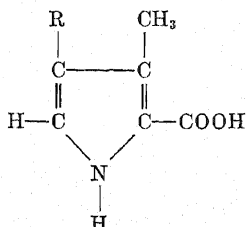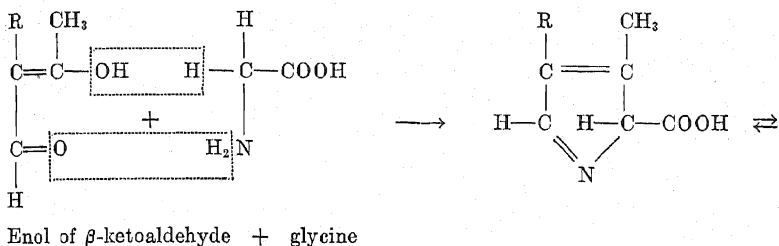
The results indicate that the nitrogen of glycine is directly employed for the synthesis of the protoporphyrin of hemoglobin, while the nitrogen of the other compounds is used indirectly, presumably by way of glycine. While the above findings prove that the nitrogen of glycine is utilized for the synthesis of a porphyrin, there seems little doubt that this conversion involves the α-carbon atom of glycine and the carboxyl carbon as well.

As glycine contains but 2 carbon atoms, other compounds probably participate with it to form the pyrrole ring. It has previously been shown that the feeding of sodium deuterioacetate to rats resulted in the formation of deuteriohemin (3). This finding showed only that some of the carbon atoms of the side chains of heme are derived from acetate, for none of the carbon atoms of the pyrrole rings is bonded to hydrogen. The feeding of compounds labeled with deuterium will therefore furnish only indirect evidence for the participation of these compounds in pyrrole ring formation.

Fischer and Fink (7) have recently shown that formylacetone and glycine readily condense to yield a product which gave the positive Ehrlich

color reaction for pyrroles. Since the product was not isolated, its structure is unknown. While insufficient data are yet available to permit definite formulation of the reactions by which porphyrins are biologically synthesized, it is possible that the synthesis involves the condensation of glycine with a β-ketoaldehyde formed in part at least from acetic acid. The reaction may occur as shown in the accompanying diagram. According to this formulation the α-carbon atom of the pyrrole rings and the carbon atoms of the methine bridges are derived from glycine.

<div align="center">EXPERIMENTAL</div>

Isotopic Nitrogen Compounds—Glycine, *dl*-glutamic acid, *dl*-leucine, and ammonium citrate containing N^{15} were synthesized by the methods previously described (8). The *dl*-proline was synthesized from isotopic potassium phthalimide and trimethylene bromide by the method of Sörensen and Andersen (9).

Feeding Experiment—A group of rats (220 to 280 gm.) was given the following diet: starch 83 per cent, yeast 5 per cent, salt mixture 4 per cent (10), cottonseed oil (Wesson oil) 6 per cent, and cod liver oil 2 per cent. After the rats were kept on this diet for 2 days, each test substance was incorporated into the diet of at least two animals for 3 days. The rats were then kept on the basal diet for the next 2 weeks. 1.33 mM of glycine and ammonia and 2.66 mM of *dl*-glutamic acid, *dl*-proline, and *dl*-leucine per 100 gm. of body weight were given over a period of 3 days.

Isolation of Hemin—The rats were killed by exsanguination. The oxalated blood was centrifuged and the red cells, after being washed twice with physiological saline, were hemolyzed with an equal volume of water. The hemoglobin solution was added dropwise over a period of 20 minutes to 3 volumes of acetic acid containing about 0.5 cc. of a saturated sodium chloride solution at 95–100°. After the addition of the hemoglobin solution, the mixture was kept on a steam bath for 1 hour. The hemin crystals were centrifuged, washed twice with a 50 per cent acetic acid solution, twice with water, twice with alcohol, and finally with ether (11).

<div align="center">SUMMARY</div>

1. Glycine has been shown to be a nitrogenous precursor of the protoporphyrin of hemoglobin in the rat.

2. The finding of N^{15} in heme after the feeding of isotopic proline, glutamic acid, leucine, and ammonia is due to the N^{15} enrichment, by the nitrogen of these compounds, of the body nitrogen from which the precursor of heme is synthesized rather than to a direct utilization of these compounds.

3. The biological formation of the porphyrin structure is briefly discussed.

BIBLIOGRAPHY

1. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159**, 567 (1945).
2. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 627 (1946).
3. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159**, 45 (1945).
4. Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 744 (1909). Lusk, G., The elements of the science of nutrition, Philadelphia and London, 4th edition, 244 (1928).
5. Ratner, S., *J. Biol. Chem.*, **152**, 559 (1944).
6. Shemin, D., *J. Biol. Chem.*, **162**, 297 (1946).
7. Fischer, H., and Fink, E., *Z. physiol. Chem.*, **280**, 123 (1944).
8. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
9. Sörensen, S. P. L., and Andersen, A. C., *Z. physiol. Chem.*, **56**, 236 (1908).
10. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
11. Nencki, M., and Zaleski, J., *Z. physiol. Chem.*, **30**, 384 (1900). Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin, 399 (1913).

THE LIFE SPAN OF THE HUMAN RED BLOOD CELL

BY DAVID SHEMIN AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

Many different methods have been used to estimate the average life time of the human red blood cell. The different attempts have yielded estimates which range from 5 to 200 days. Estimates have been made by inducing polycythemia and noting the time required for the return to the normal red cell count; this yielded values of 18 to 30 days (1). Determinations of the amount of iron or pigment excreted have yielded estimates of 20 to 200 days (2).

Determinations of the survival time of transfused erythrocytes by means of differential agglutination (3, 4) yielded estimates ranging from 30 to 100 days. The use of agglutinogens M and N for tagging erythrocytes gave values of 80 to 120 days (5-7). More recently Callender, Powell, and Witts (8) using Ashby's differential agglutination method (3) in Rh-positive men (9) concluded from a mathematical analysis of their data that red blood cells live for approximately 120 days. Measurement of the time required for the disappearance of sulfhemoglobin from the blood of cyanosed workers (10) indicated the life span of the red blood cell to be 115 days. Hawkins and Whipple (11) by another technique obtained a value of 124 days for the dog erythrocyte.

Glycine has been shown to be a nitrogenous precursor of the protoporphyrin of hemoglobin in the rat (12). The feeding of glycine labeled with N^{15} to a man also results in the formation of heme containing a comparatively high concentration of N^{15} (13). After cessation of the feeding of the labeled glycine, the isotope concentration of the heme was followed over a long period of time by analysis of the hemin isolated at intervals. The values rose rapidly to a high level, remained practically constant for many weeks, and then fell quite sharply to a very low level. This finding indicates that the heme is neither involved in the dynamic metabolic state nor reutilized for hemoglobin formation. On these grounds, the curve of N^{15} concentration of the heme *versus* time can form the basis for a determination of the average life span of the human red blood cell. This was found to be about 127 days.

EXPERIMENTAL

Feeding Experiment—One of us (D. S.) ingested 66 gm. of glycine containing 32.4 atom per cent N^{15} excess (14). The glycine was divided into 60

doses and taken over a period of 3 days.   At intervals blood was withdrawn
and urine collected.

Preparation of Hemin and Protein Samples—Usually 20 cc. of venous
blood were withdrawn, oxalated, and separated into red cell and plasma

TABLE I

N^{15} *Concentration of Hemin*

Time	Hemin N^{15} concentration	Time	Hemin N^{15} concentration
days	*atom per cent excess*	*days*	*atom per cent excess*
0	0.000	127	0.342
4	0.134	144	0.200
18	0.422	154	0.164
77	0.466	170	0.112
86	0.462	192	0.096
99	0.448	231	0.062
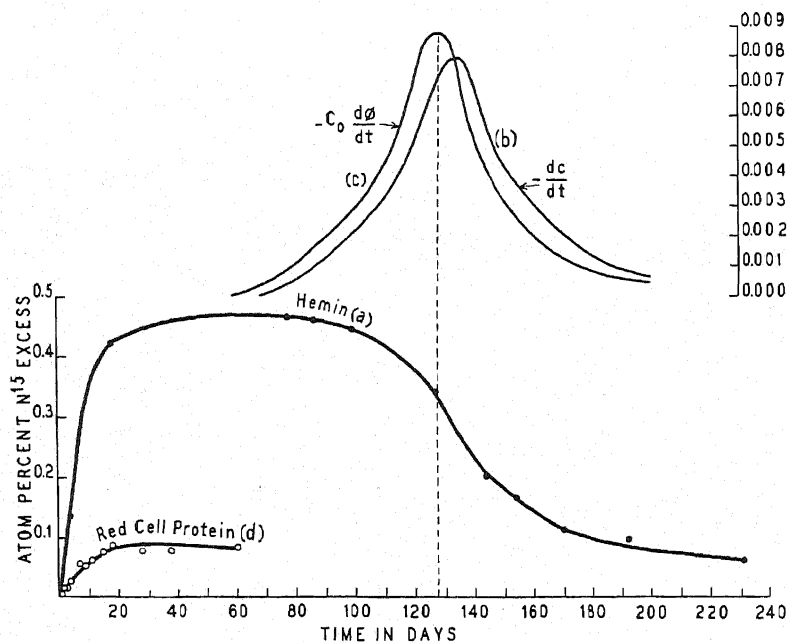
FIG. 1. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 3 days

fractions by centrifugation.   On the 4th and 18th days of the experiment
300 cc. quantities of blood were withdrawn.   These larger samples were
taken to permit isolation of individual amino acids from the blood proteins.
The results obtained on the amino acids and proteins will be reported in a
later publication.

The hemin was isolated in a manner previously described (15). The red blood cell proteins were obtained by treating hemolyzed red cells with trichloroacetic acid.

Urinary Urea Nitrogen—The urinary urea nitrogen was obtained as ammonia by treating urine, from which ammonia had been adsorbed with permutit, with urease.

Isotope Concentrations—The isotope concentrations found in the hemin at various periods of the experiment are given in Table I. For comparison there are plotted in Fig. 1 the isotope concentrations found in the hemin (Curve *a*) and red cell proteins (Curve *d*).

DISCUSSION

If during the administration of an isotopic compound a nitrogenous constituent of a cell is continuously being degraded and resynthesized, though morphologically the cell remains intact, the N^{15} concentration will increase to a maximum value, and then decline. The average life span of the cell cannot be thus determined but only the half life time of the constituent of the cell, which can be estimated from the rate of decrease of the N^{15} concentration of the particular component (16, 17). This problem has been mathematically treated by Zilversmit, Entenman, and Fishler (18).

The shape of the curve in which the isotope concentration of the hemin is plotted against time is different from that found previously for any cellular constituent. Instead of rising during the period in which the labeled material is fed and then decaying along an exponential curve, the curve for hemin rises rapidly for about 25 days after the cessation of feeding the labeled glycine, remains practically flat for the next 70 days, and then falls along an S-shaped curve. Such a curve cannot be the result of a single process involving molecules which are synthesized and degraded by a random procedure, a process which would result in a curve similar in shape to that we found for the liver proteins of the rat (17). The curve for hemin cannot be described by a simple exponential function; it appears that the probability that a hemoglobin molecule may be degraded is a function of its age. Since the concept of "aging" is not applicable to a molecule, another explanation must be sought.

If a constituent of a cell is not involved in the flux of synthesis and degradation, the presence of N^{15} in the constituent of this cell must be the result of synthesis of the component and its incorporation during the formation of the cell. The original molecules of this constituent will then remain with the cell until the cell disintegrates or dies. In such a case the N^{15} concentration of the component will, if the cells are not indiscriminately destroyed, reach a maximum value and remain constant for a length of time depending on the life span of the cell. On the destruction of the cell the N^{15} concen-

tration should then, unless the component is reutilized, abruptly decline. In such a system, the average life time of the cell, as will be shown below, can be estimated from the curve obtained by plotting the N^{15} concentration of the component against time. During the period in which the labeled compound was fed in this experiment, heme containing isotopic nitrogen was synthesized and incorporated into newly formed erythrocytes. These cells as they are discharged into the circulation raise the isotope concentration of the heme of the total red blood cells. Up to about the 25th day the rise takes place rapidly. After this period, and probably even earlier, the isotope concentration of the newly synthesized heme is lower than that of the average N^{15} concentration of the heme in circulation. The addition of this heme of relatively low isotope concentration to the circulating heme would lower the average isotope concentration if the destruction of heme were a random process. However, the molecules to be destroyed are those in the red blood cells which had been synthesized before feeding the labeled glycine. As these contain no excess N^{15}, the isotope concentration of the heme in the red cells rises, even though the newly added material has a lower isotope concentration than the average circulating heme. Eventually the time arrives at which the red cells, synthesized during the period in which labeled glycine was fed and which contain heme with the highest isotope concentrations, begin to be destroyed. As the bulk of these red cells is replaced by others containing heme with very low isotope concentration the N^{15} concentration in the total heme drops abruptly. This decrease of N^{15} concentration begins at about the 80th day. This phenomenon can occur only if the porphyrin moiety of hemoglobin is not reutilized for new hemoglobin formation. Were the heme used again, the isotope concentration of the isolated hemin would decrease very slowly instead of showing an abrupt drop. In this respect the protoporphyrin of the hemoglobin differs from the iron, which is reutilized after the destruction of the red cell (19, 20).

These considerations indicate that the heme of the non-nucleated red blood cells, unlike the constituents of nucleated cells, is not continuously formed and degraded within the cell. The red cells are not indiscriminately destroyed, but their rate of destruction is a function of the age of the cell.

A mathematical treatment of the data gives a figure of the average life time of the red cell of 127 days, which is the same time as that separating the mid-points of the rising and declining portions of the curve.

An equation relating the isotope concentration $C(t)$ of the hemin as a function of time is derived below.

Let

$f(\theta)$ = the N^{15} concentration in the hemin synthesized at the time θ. $f(\theta)$ is also the isotope concentration in the hemin precursor at time θ

a = rate of formation of hemin nitrogen in atoms per day
N = total hemin nitrogen (atoms) in the blood
$G(t)$ = atoms of N^{15} in circulating hemin nitrogen at time t
$\phi(t)$ = probability that a red cell will have a life span greater than t

Then

$$G(t) = \int_0^t af(\theta)\phi(t - \theta)d\theta \tag{1}$$

Since

$$100\,\frac{G(t)}{N} = C(t) \tag{2}$$

$$C(t) = 100\,\frac{a}{N}\int_0^t f(\theta)\phi(t - \theta)d\theta \tag{3}$$

Let

$$\frac{N}{a} = T$$

Then

$$C(t) = \frac{100}{T}\int_0^t f(\theta)\phi(t - \theta)d\theta \tag{4}$$

There does not exist a general solution of this integral equation.[1] An approximate solution may be obtained as follows: Assume $f(\theta)$ is zero every place except at $t = 0$ and that $\int f(\theta)d\theta$ is finite. Physically this assumption concerning $f(\theta)$ is equivalent to the sudden introduction into the circulation at zero time of newly formed red cells whose hemin is labeled with N^{15}. Equation 4 becomes

$$C(t) = \frac{100}{T}\phi(t) \tag{5}$$

and

$$\frac{dC}{dt} = \frac{100}{T}\frac{d\phi}{dt} \tag{6}$$

From the definition of ϕ it is apparent that $-d\phi/dt$ is proportional to the rate of destruction of red cells. Therefore under these conditions the rate of destruction will also be proportional to $-dC/dt$.

We have graphically evaluated $-dC/dt$ for the period $t > 80$ days. These values are plotted in Fig. 1, Curve b. This curve shows that cells formed during the period in which high concentration isotope was being incorporated into red cells, i.e. the 3 day feeding period, begin to die at

[1] A treatment of the Volterra equation is given by Margenau and Murphy (21).

about the 70th day. Therefore, from $t = 0$ to $t = 70$, $\phi(t)$ must be constant at the value 1. $- d\phi/dt$ attains its maximum value at $t = 133$ days. As a first approximation this may be taken to be the average life span of the red cells, $\overline{T}$. During the initial period, certainly up to $t = 30$, $\phi(t)$ is constant at unity; i.e., all cells survive for more than 30 days. During this period when $\phi(t - \theta)$ is equal to 1, equation 4 can be simplified to

$$C(t) = \frac{100}{\overline{T}} \int_0^t f(\theta)d\theta \qquad (7)$$

Differentiating (Equation 7) with respect to t, we get

$$\frac{\overline{T}}{100} \frac{dC}{dt} = f(t) \qquad (8)$$

If we take 133 days to be the value of $\overline{T}$, we can from the slope of the initial portion of the curve calculate $f(\theta)$, the concentration of the nitrogenous precursor of hemin. We have not attempted this since our experimental values in this region ($t = 0$ to $t = 30$) are too scanty to define the curve precisely. Further, this value of $\overline{T}$ is certainly too large, since the labeled red cells have been formed over a period of time and not at an instant. To correct for this factor the initial portion of the curve must be considered.

Returning to the general equation (No. 4), let

$$x = t - \theta$$

$$dx = -d\theta$$

Then

$$C(t) = -\frac{100}{\overline{T}} \int_t^0 f(t - x)\phi(x)dx = \frac{100}{\overline{T}} \int_0^t f(t - x)\phi(x)dx \qquad (9)$$

As mentioned before, for an arbitrary function $f(t - x)$ Equation 9 cannot be generally solved. The initial part of Curve a, Fig. 1, can, however, be fitted with sufficient accuracy up to the 30th day by an equation of the form

$$C(t) = C_0(1 - e^{-\lambda t}) \qquad (10)$$

with $C_0 = 0.48$ and $\lambda = 0.11$.

Then

$$\frac{dC}{dt} = C_0 \lambda e^{-\lambda t} \qquad (11)$$

It follows from Equations 8 and 11 that

$$f(t) = \frac{\overline{T}}{100} C_0 \lambda e^{-\lambda t} \tag{12}$$

Substituting this into Equation 9 yields

$$C(t) = \int_0^t C_0 \lambda e^{-\lambda(t-x)} \phi(x)\,dx \tag{13}$$

$$C(t) = C_0 \lambda e^{-\lambda t} \int_0^t e^{\lambda x} \phi(x)\,dx \tag{14}$$

Differentiating with respect to t yields

$$\frac{dC}{dt} = -\lambda \cdot C(t) + C_0 \lambda \cdot \phi(t) \tag{15}$$

$$C_0 \cdot \phi(t) = \frac{1}{\lambda}\frac{dC}{dt} + C(t) \tag{16}$$

$$C_0 \frac{d\phi}{dt} = \frac{1}{\lambda}\frac{d^2 C}{dt^2} + \frac{dC}{dt} \tag{17}$$

Equation 17 shows that the death rate of the cells is not proportional to $-dC/dt$ as we initially assumed, but that another factor $(1/\lambda)(d^2C/dt^2)$ must be added to correct for the fact that the labeled red cells were not generated in an instant but have been produced over an extended interval of time, though with a rapidly declining isotope concentration.

By a graphical procedure we have evaluated d^2C/dt^2 and have used Equation 17 to compute values of $-C_0(d\phi/dt)$ for values of the argument between $t = 80$ to $t = 220$; the result is shown in Fig. 1, Curve c. In confirmation of our earlier assumption $d\phi/dt$ is found to be zero, $i.e.$ $\phi(t) = 1$, from $t = 0$ to $t = 70$. The maximum value of $-C_0(d\phi/dt)$ occurs at $t = 127$ days. Since the death curve $-(d\phi/dt)$ $versus$ time is seen to be symmetrical about the ordinate $t = 127$, the average life span of the red cell must also be 127 days.

If half the red cells have a life span between $\overline{T} - \Delta$ and $\overline{T} + \Delta$, then

$$\frac{\int_{\overline{T}-\Delta}^{\overline{T}+\Delta} \left(-\frac{d\phi}{dt}\right) dt}{\int_0^\infty \left(-\frac{d\phi}{dt}\right) dt} = \frac{1}{2}$$

By graphical integration of the curve $-C_0(d\phi/dt)$ $versus$ time, Δ is found to be 14 days. This means that half the red cells survive for them 113 to 141 days, while the other half die before and after this period.

Variations in the value of C_0 and λ have but small effect upon the value of $\overline{T}$. The value for the average life span of the red cells, which is presumably much the same for all normal male human adults, corresponds to the production (and destruction) of 0.79 per cent of red cells per day. The value of $\overline{T}$ only relates to the period the red cell is in the circulatory system. The period between the introduction of heme into the red cell and its discharge into the circulation cannot exceed a few days. On the 4th day after the beginning of the experiments the red cells in the blood contained an appreciable concentration of labeled heme. It is likely that the time between the period in which the heme is formed and the moment it appears in the circulation cannot exceed 1 to 2 days.

By the isotope technique here described, in contrast to the other techniques, the life span of the red cells can be determined in the individual in which they are produced and destroyed under physiological conditions.

With this knowledge of the life span of the red blood cell it is possible to confirm the fact that glycine is the precursor of the protoporphyrin of hemoglobin in the human as well as in the rat. 10 days from the start of the experiment the N^{15} concentration of the protoporphyrin was 0.34 atom per cent N^{15} excess. Since the average life time of the red blood cell is about 127 days, approximately one-thirteenth of the cells are, at this time, newly formed and contain isotopically labeled heme. The newly formed red cells must contain heme having an average N^{15} concentration 13 times as high as the total heme or 4.4 atom per cent N^{15} excess (*i.e.* 13 $\times$ 0.34). The nitrogenous source of the heme in this 10 day period, therefore, must have had an average N^{15} concentration of 4.4 atom per cent N^{15} excess. It is clear from quantitative considerations that glycine, the isotopic amino acid fed, is the only compound that could have had as high an average N^{15} concentration for the first 10 days. It is known that ammonia and glutamic acid have the next highest N^{15} concentrations. Ammonia can easily be eliminated. The N^{15} concentration of excretory urea, which is derived from ammonia, was determined through this period, and found to be far below the required 4.4 per cent. The isotope concentration in the urinary urea rose rapidly to a maximum concentration of 3.3 atom per cent excess on the 3rd day of the experiment and then rapidly fell to 0.37 atom per cent excess on the 7th day of the experiment and had fallen to 0.188 atom per cent excess by the 10th day. The average isotope concentration in the urinary urea was less than 1.2 atom per cent excess during this period. Glutamic acid can also be eliminated. The N^{15} concentration of glutamic acid is always found to be approximately equal to that found for the whole protein, for glutamic acid nitrogen rapidly equilibrates with most of the nitrogen of the protein (17). Even the plasma proteins, which are among those with the highest N^{15} concentration after the feeding

of an isotopic compound, had an average N^{15} concentration far below 4.0 atom per cent N^{15} excess. The maximum isotope concentration attained by the plama proteins was only 0.39 atom per cent excess.

The finding that the isotope concentration in the red cell proteins, which consists mainly of globin, are low (Fig. 1, Curve d) is consistent with the view that the proteins, like the heme, of the erythrocytes differ from the proteins of other organs in not being involved in the dynamic state. The red cell, when formed, is supplied with its store of hemoglobin, which remains intact during its lifetime. From this it may be inferred that only about 0.79 per cent of the globin is synthesized per day.[2]

We are indebted to Dr. Chaim L. Pekeris of Columbia University, Mathematical Physics Group, for helpful discussions and assistance in the mathematical portion of this paper.

SUMMARY

1. A study of the isotope concentrations found in the heme of the human red blood cell, after the feeding of glycine labeled with N^{15}, indicates that the erythrocyte is not subjected to indiscriminate destruction but has a life span. This was found to be about 127 days.

2. Evidence has been presented which shows that the protoporphyrin of hemoglobin is not reutilized for hemoglobin synthesis.

3. Glycine is the nitrogenous precursor of the protoporphyrin of hemoglobin in man.

BIBLIOGRAPHY

1. Escobar, R. A., and Baldwin, F. M., *Am. J. Physiol.*, **107**, 249 (1934).
2. Schiødt, E., *Acta med. Scand.*, **95**, 49 (1938).
3. Ashby, W., *J. Exp. Med.*, **29**, 267 (1919); **34**, 127 (1921).
4. Wearn, J. T., Warren, S., and Ames, O., *Arch. Int. Med.*, **29**, 527 (1922).
5. Landsteiner, K., Levine, P., and Janes, M. L., *Proc. Soc. Exp. Biol. and Med.*, **25**, 672 (1928).
6. Wiener, A. S., *J. Am. Med. Assn.*, **102**, 1779 (1934).
7. Mollison, P. L., and Young, I. M., *Quart. J. Exp. Physiol.*, **30**, 313 (1940); **31**, 359 (1941–42).
8. Callender, S. T., Powell, E. O., and Witts, L. J., *J. Path. and Bact.*, **57**, 129 (1945).
9. Landsteiner, K., and Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, **43**, 223 (1940).
10. Editorial, reference to Jope, unpublished work, *Brit. Med. J.*, **1**, 576 (1946).
11. Hawkins, W. B., and Whipple, G. H., *Am. J. Physiol.*, **122**, 418 (1938).
12. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621 (1946).
13. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159**, 567 (1945).
14. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).

[2] In an experiment in which labeled histidine was fed to rats it was found that about 1 per cent of the globin was synthesized per day (private communication from Dr. C. Tesar).

15. Nencki, M., and Zaleski, J., *Z. physiol. Chem.*, **30**, 384 (1900). Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin, 399 (1913).
16. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, **144**, 545 (1942).
17. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **153**, 401 (1944).
18. Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, **26**, 325 (1942–43).
19. Cruz, W. O., Hahn, P. F., and Bale, W. F., *Am. J. Physiol.*, **135**, 595 (1941–42).
20. Hahn, P. F., Bale, W. F., and Balfour, W. M., *Am. J. Physiol.*, **135**, 600 (1941–42).
21. Margenau, H., and Murphy, G. M., The mathematics of physics and chemistry, New York, 503 ff. (1944).

REVERSIBLE INACTIVATION OF THYROTROPIC HORMONE BY ELEMENTAL IODINE

I. THE ACTION OF IODINE*

By ALEXANDER ALBERT, RULON W. RAWSON, PRISCILLA MERRILL, BEATRICE LENNON, AND CHARLOTTE RIDDELL

(From the Thyroid Clinic, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston)

The mechanism by which the thyrotropic or the thyroid-stimulating hormone (TSH) causes such marked growth and accelerated function of the thyroid remains a perplexing problem. Significant experiments in this direction have been reported by Rawson *et al.* (6, 8) who exposed TSH to slices of various organs in tissue culture. Of the various tissues studied, only thyroid, thymus, and lymph node were capable of inactivating an appreciable amount of hormone. In the case of thyroid tissue, it was observed that one rabbit thyroid weighing about 150 mg. was capable of inactivating 12 units of thyrotropic hormone. Furthermore, slices of human thyroids obtained at operation varied in their ability to inactivate a given amount of hormone; *i.e.*, slices of toxic goiters were twice as effective as equal amounts of normal tissue. Although the mechanism and meaning of this inactivation are obscure, one possibility is that in exerting its effect on the thyroid TSH may be partly metabolized. Such an inactivation could be carried out by an enzyme of the thyroid or by some substance formed by the thyroid (Rawson *et al.* (8)).

The thyroid possesses the unique ability of trapping circulating iodide and presumably converting it into iodine. Subsequently, the formation of thyroxine, which is the ultimate function of the thyroid, is possible. How TSH is integrated with this system is unknown, since the enzyme which supposedly converts iodide to iodine is purely theoretical and since no evidence is available showing any biochemical interrelation of TSH with any of the products of the thyroid. It would seem that the oxidation of iodide is a prime function of the thyroid. It is known that iodide has a depressing effect on the ability of concurrently administered TSH to induce goiters, and that iodide has an involuting action on the hyperplastic goiters of Graves' disease and on the hyperplastic goiters induced by some antithyroid drugs.

In view of these considerations, it was thought worth while to investigate the action of iodine on TSH.   The experiments which follow concern only the *in vitro* effect of iodine on TSH extract and as such do not necessarily reflect any physiological significance.   We will show that iodine abolishes TSH activity at room temperature within 30 minutes by the formation of an iodo complex of TSH 'extract, and that the inactivation is reversible. In a subsequent paper, we will show that the reactivation of TSH is accomplished instantaneously by several reducing agents, particularly those which are goitrogenic.

Materials and Methods

Thyrotropic hormone was generously supplied by Parke, Davis and Company in the form of 10 ml. ampules of a sterile aqueous solution containing 50 Junkmann-Schoeller units per ml.   The activity of this pituitary extract was 2.5 units per mg. of protein.   All hormone used was prepared from the same batch (No. 098198-A).   It should be emphasized that this TSH preparation is an impure pituitary extract containing TSH and other active as well as inert proteins.

Our assay for thyrotropic hormone is a composite modification of three methods.   Ten to twenty, 1 day-old cockerels (white Leghorn) were injected once daily with a volume usually of 1 ml. subcutaneously for 3 days, and killed on the 4th day.   The thyroid glands were dissected and weighed *en masse* on a torsion balance.   After weighing, the glands were placed in 10 per cent KOH and iodine determinations made according to the method of Kendall, as modified by Astwood and Bissell (1).   Finally, some of the thyroids were sectioned and the mean acinar cell height determined by the method of Rawson and Salter (7) modified so that 50 cells were measured in each of five thyroid lobes of five chicks.

Interpretation of the methods of assay of TSH is very important and deserves some detailed comment.   Fig. 1 shows the effect of graded doses of TSH on the weight, iodine concentration, and mean cell height of the chick thyroid.   These results are confirmatory of findings previously reported by Smelser (9), Bergman and Turner (2), Stimmel and McCullagh (10), and others (5).   Such values are useful for assay purposes only between a range of 0 to 2 units of TSH because the values for weight and mean cell height become rapidly asymptotic with doses of hormone greater than 2 units.   It can also be seen that the thyroidal iodine concentration is a more sensitive indicator of TSH than either mean cell height or weight of the thyroid.   Consequently, we have calculated all our results by reading off the unitage from the weight curve.   All crucial experiments were checked by the mean cell height curve.   Iodine concentrations are given in each case and are used only to indicate the positivity of the value obtained from

the weight curve, but not necessarily the accuracy of this value because of two reasons. The first is obvious from the curves, because the iodine loss in the range of dosage between 0.5 and 2 units is maximal. The other reason is that in most of the experiments exogenous iodine was given concurrently with TSH. From several hundred assays, it can be stated, that, provided no iodine is given concurrently with TSH, hyperplasia and hypertrophy of the thyroid do not occur without an intensive and concomitant or previous fall in iodine concentration. With exogenous iodine, the fall in
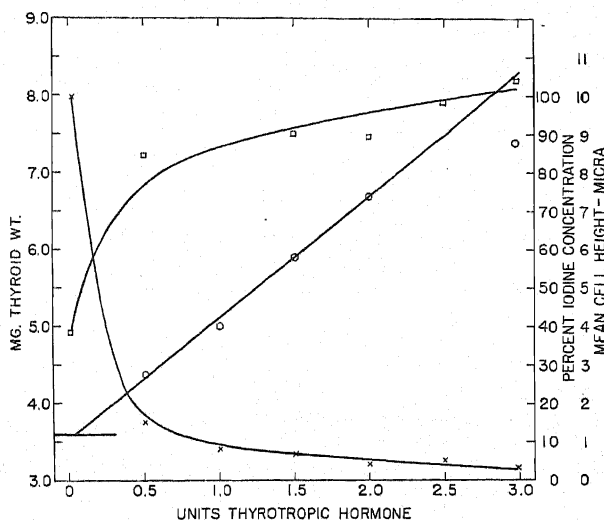
FIG. 1. The assay of thyrotropic hormone as based on the relationship between dosage and thyroid weight, thyroid iodine concentration, and mean cell height. Each point on the curves for thyroid weight and iodine concentration represents ten animals. Each mean cell height value is an average of 50 measurements on each of five thyroid lobes of five different animals at any given dosage. □ values of mean cell height, O average thyroid weights, X iodine concentration values. The horizontal line at the lower left represents the range of thyroid weights of untreated chicks, 4 days of age.

concentration is less but the general rule applies. This is probably due to the fact that iodine loss is partly balanced by the increased iodine avidity of the gland rendered hyperplastic by TSH. In any case, iodine concentration in such a situation cannot be used as a quantitative index for TSH. It is significant that all three methods of assay check well, as can be seen in Tables I to VIII, especially Table VII containing the mean cell heights.

The exact values (Fig. 1) of the weights obtained with various amounts of TSH are not necessarily observed if the series is repeated each month. The curves, however, are parallel to that shown in Fig. 1, and on the same

scale fall either to the right or left of it. This probably indicates the inherent variation in sensitivity of different batches of animals. To compare results from week to week, a group of uninjected animals and a group injected with a certain amount of hormone, usually either 1 or 2 units, depending on the purpose of the experiment, are always included. The actual unitage is read off the curve by the average weight induced, as are all other values of any one series. The differences among all experimental values are then expressed as percentage change of determined control value, and not from the assumed amount given. Thus, similar experiments performed at widely separated intervals can be directly compared.
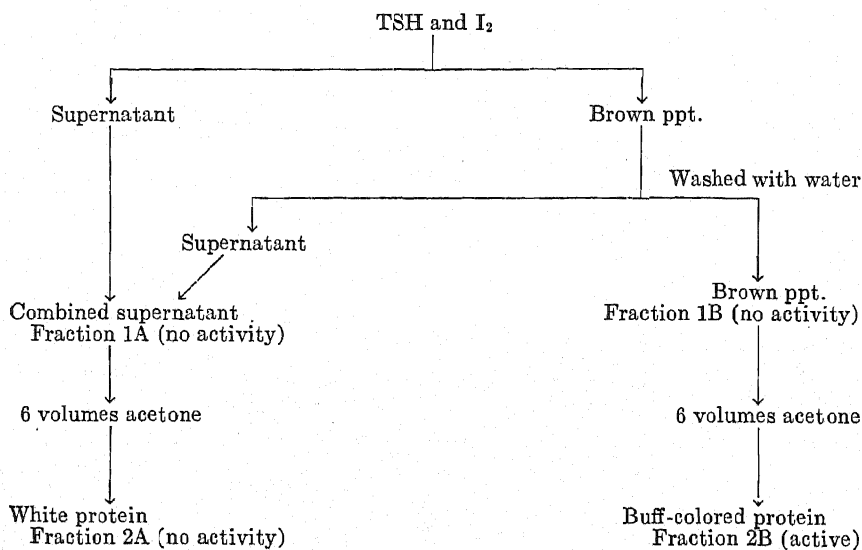
Over a dozen units have been described for thyrotropic hormone. It is not possible in some cases to convert to a common basis the results among investigators interested in either potency of the hormone, isolation procedures, or physiological experiments. As a step in this direction, the "international standard preparation of the anterior lobe of the ox"[1] was assayed by the method just described and was found to contain 1 Junkmann-Schoeller unit per 15 mg.

Results

Effect of Iodination on Thyrotropic Activity—To 1.0 ml. of TSH extract containing 20 mg. of protein were added 2.0 ml. of I_2-KI solution (1 per

---

[1] Obtained through the courtesy of Dr. Adley B. Nichols, Committee of Revision of the Pharmacopoeia of the United States of America, Philadelphia.

cent I_2 in KI) and 7.0 ml. of water. After standing for 30 minutes at room temperature (24°), a deep brown precipitate formed. The precipitate was removed by centrifuging, washed with 10 ml. of water, and recentrifuged. An aliquot from the combined supernatant fluids and from the emulsified precipitate was removed for assay, and the remaining materials poured into 6 volumes of cold acetone. The resulting white precipitates thus formed were removed, dissolved in distilled water, and assayed. The results are presented in the accompanying flow sheet and in Table I. In all cases, the assays were conducted at a level of 2 units per animal.

TABLE I

Effect of Iodination and Partial Deiodination on Thyrotropic Activity

Assay No.	Treatment of extract	Thyroid weight
		mg.
A-3	Uninjected controls	3.5
A-4	0	7.7
A-0	*	8.9
A-1	As Fraction 2A	4.0
A-2	" " 2B	7.5
A-13	Uninjected controls	3.0
A-8	0	9.1
A-11	As Fraction 1B	3.7
A-9	" " 2B	9.6
A-12	" " 1A	3.2
A-10	" " 2A	3.2

* 20 mg. of protein mixed with 20 mg. of I_2. After precipitation had occurred, the entire mixture was poured into 6 volumes of acetone, and the acetone precipitate removed and assayed.

The iodine partition during the iodination of the extract was demonstrated by mixing 100 units of TSH (no demonstrable I) with 40 mg. of I_2 in KI. The washed brown precipitate was found by analysis to contain a total of 4 mg. of iodine. When the precipitate was washed with excess acetone, the resulting buff-colored product contained a total of 1.6 mg. of iodine. On the basis of activity, it can be calculated that 40 γ of iodine are present in the iodinated complex per unit of original TSH activity. 24 γ are readily removed by acetone, leaving 16 γ of iodine per unit of TSH activity. The presence of 16 γ of I which appears more tightly bound to the proteins of the extract does not inhibit TSH activity.

Effect of Amount of Iodine on Inactivation of TSH Extract (Hormone Constant, Iodine Variable)—25 units of TSH were mixed with varying amounts of I_2 in KI. When precipitates formed after 1 hour, the mixtures were diluted to 37.5 ml. with distilled water, and assayed at the

equivalent of 2 original TSH units.   It can be seen (Table II) that 100 γ
of I₂ per unit of TSH constituted the minimal amount of iodine inducing
maximal inactivation.   It can also be seen that, despite the large doses
of total iodine the chicks received, the percentage iodine loss of their

TABLE II

*Effect of Increasing Amounts of Iodine on Constant Amount of Thyroid-
Stimulating Hormone (TSH)*

Assay No.	Units TSH	I₂ in KI	Total I per chick	Thyroid weight	Thyroid I	Average mean cell height	Per cent inactivation
		mg.	*mg.*	*mg.*	*γ per mg.*	*μ*	
22	25	10.0	1.6	4.50	0.58	4.5	89
23	25	5.0	0.8	4.50	0.62	5.4	89
24	25	2.5	0.4	4.40	0.54	5.4	91
25	25	1.0	0.16	5.75	0.41	9.8	59
26	25	0.5	0.08	7.40	0.38	10.8	22
27	25	0.0	0.0	8.30	0.14	10.8	0
28	0	0.0	0.0	4.00*	0.85	4.5	

* The control value is higher and the I value lower than usual, since this series
was made with 5 day-old chicks.

TABLE III

*Effect of Increasing Amounts of TSH and Iodine in Constant Ratio
(400 γ of I₂ per Unit of TSH)*

Assay No.	Units TSH	I₂	Units per chick	Total I per chick	Thyroid weight	Thyroid I	Per cent inactivation
		mg.		*mg.*	*mg.*	*γ per mg.*	
32	0	0	0	0	3.2	1.80	
33	30	0	2	0	7.0	0.38	0
34	50	20	5	2	7.8	0.94	60
35	100	40	10	4	8.8	1.26	75*
36	250	100	25	10	10.8	1.48	85*
37	500	200	50	20	6.6	0.70	95

* The values are very approximate and calculated as if the linear relationship
between thyroid weight and TSH holds above 8.0 mg., which it does not.

thyroids was proportional to the remaining hormonal activity, but at a
slightly lower level.

*Effect of Increasing TSH Extract and Iodine (Amounts of Hormone and
Iodine Both Variable, Ratio Constant)*—Hitherto, all assays were performed
at a level of 2 units per chick, and at this level, maximal inactivation (90 per
cent) was obtained.   It was significant to determine whether similar in-
activation could be obtained when much larger dosages were tested.   Con-
sequently mixtures of TSH and I₂ were prepared so that each chick was

injected with dosages varying from 5 to 50 units of the original TSH.   In
each case, 400 γ of iodine in KI were added per unit of TSH.   It

TABLE IV

Effect of Constant Amount of Iodine on Increasing Amounts of TSH

Assay No.	Units TSH	I₂ in KI	Units per chick tested	Total I per chick	Thyroid weight	Thyroid I	Per cent inactivation
		ml.		*mg.*	*mg.*	γ *per mg.*	
154	30	15	2	0.2	3.25	1.21	100
130	0	0	0	0.0	2.94	0.95	
131	30	0	2	0.0	7.26	0.07	0
132	30	15	2	0.2	3.55	0.89	100
196	20	30	2	0.2	3.25	0.88	100
197	50	30	5	1.0	3.84	0.83	100
198	100	30	10	2.0	4.50	0.87	95
199	200	30	20	4.0	4.90	0.82	95
200	200	0	20	0.0	9.60	0.03	0
195	0	0	0	0.0	3.19	1.10	

TABLE V

Effect of Iodine in Various Forms on Activity of Constant Amount of TSH

Assay No.	Units TSH per chick	Total iodine per chick	Type of iodine	Thyroid weight	Thyroid I
		mg.		*mg.*	γ *per mg.*
70	0	0	0	3.2	1.20
71	2	0	0	6.3	0.24
72	2	0.01	KI	6.4	0.34
73	2	0.05	``	7.5	0.84
74	2	0.10	``	8.0	0.91
76	2	1.00	``	7.4	0.26
77	2	10.00	``	8.9	1.15
78	2	0.01	I₂ in KI	8.4	0.74
80	2	0.10	`` `` ``	9.6	0.77
81	2	0.50	`` `` ``	8.9	0.69
82	2	1.00	`` `` ``	6.5	1.37
83	2	10.00	`` `` ``	4.4	1.30
84	2	0.01	``	9.7	0.25
85	2	0.05	``	8.1	0.47
86	2	0.10	``	8.8	
87	2	0.50	``	7.1	0.52
88	2	1.00	``	6.8	1.00
89	2	10.00	``	4.8	1.31

can be noted (Table III) that the resulting inactivation was roughly in-
dependent of the level at which the TSH is tested.

Effect of Increasing TSH Extract (Hormone Variable, Iodine Constant)—
To a great excess of iodine varying amounts of TSH extract were added.
The resulting precipitates were removed, emulsified in water, and tested.
Chicks received dosages varying from 2 to 20 units (Table IV). Under
these conditions, almost complete inactivation was observed.

TABLE VI

Effect of Iodine Alone in Various Forms on Thyroid

Assay No.	Total iodine per chick	Type of iodine	Thyroid weight	Thyroid I
	mg.		*mg.*	*γ per mg.*
130	0	0	2.94	0.95
138	0.01	KI	3.40	1.30
139	0.10	"	4.12	1.50
140	1.00	"	4.55	1.03
141	10.00	"	4.34	1.01
142	0.01	I_2 in KI	3.57	1.13
143	0.10	" " "	3.78	1.15
144	1.00	" " "	3.96	1.01
145	10.00	" " "	4.16	1.06
146	0.01	"	3.50	1.05
147	0.10	"	4.00	1.33
148	1.00	"	3.90	1.53

TABLE VII

Survey of Activity by Microhistological Assay

Assay No.	Agents per chick	Average mean cell height
		μ
221	800 γ TSH	9.08
222	800 " iodinated TSH	3.82
223	800 " " " after acetone	7.74
224	800 " TSH + 200 γ I as KI	8.30
225	200 " I as KI	3.87
226	200 " " " I_2	3.77
227	200 " " " I_2-KI	4.45
195	None	3.86

Effect of Chemical Form of Iodine—Since it is well known that iodine in
almost any form has a depressing effect both on the growth and function
of the thyroid, it was evident that the biological or *in vivo* effect of iodine
had to be determined. Since inorganic, and perhaps also organic, iodine
reaches the thyroid as iodide, the effect of the same dosages of iodine as
(1) iodide, (2) iodine in iodide, and (3) iodine alone on the activity of TSH

was determined. Solutions used were KI (10 mg. of iodine as KI per ml.), dilute Lugol's solution (10 mg. of total iodine per ml. as I_2 in KI, 1 part of I_2 to 2 parts of I as KI), and an alcoholic solution of I_2 (10 mg. of I_2 per ml.). 30 units of TSH were mixed with the various iodine solutions and dilutions made so that each chick received 2 units of TSH and varying amounts of iodine from 10 to 10,000 γ (Table V). Controls consisted of animals not injected, injected with 2 units of TSH alone, and injected with varying dosages of the three types of iodine alone, the latter being shown in Table VI. The chief conclusion was that KI did not precipitate TSH extract nor did it interfere with TSH activity, while both I_2 in KI and I_2 alone did. Iodine in any of the three forms used had no effect on the thyroid.

DISCUSSION

It has been shown that elemental iodine abolished TSH activity. The inactivation is dependent upon the amount of iodine added and does not appear to occur all at once when a certain concentration of iodine is reached. The reaction mixture must contain a slight excess of free iodine. Furthermore, the inactivation is induced no matter how high a level of TSH is tested, provided sufficient iodine is present. The greatest percentage of inactivation is induced, however, when TSH extract in either small or large doses is added to a great excess of iodine.

Several possibilities could account for the abolition of TSH activity by iodine. The first is that the ability of the chick thyroid to respond to TSH is abolished by the excess iodine present in the iodinated complex. However, iodide in various doses does not affect the goitrogenic ability of a given amount[2] of the hormone, nor does it form an insoluble iodinated protein complex with the TSH extract. The second possibility is that during iodination of TSH extract by elemental iodine a certain amount of thyroxine can be formed. 2 units of TSH were usually used and this amount of activity contains approximately 800 γ of protein. Assuming a generous yield of 8 per cent, each animal would receive about 60 γ of thyroxine, which could account for the observed inactivation of TSH by general depression of the thyroid's ability to respond to TSH (Cortell and Rawson (4)). There are several arguments against such a possibility, since the formation of thyroxine in acid medium is not favorable, since acetone restores activity fully but removes only part of the iodine, and since the inactivation does not parallel the absorption of iodine by the protein but occurs when excess free iodine is present. This point was tested experimentally by injecting 60 γ of thyroxine (synthetic) with TSH (Table VIII).

[2] In these experiments, this statement pertains only to a dosage of 2 units TSH per chick.

It is apparent that while some loss of TSH potency occurred the loss was hardly sufficient to account for the inactivation of TSH by iodine via the production of thyroxine on iodination of the proteins of the extract.

A third possibility is that, since the TSH used was quite impure, the remaining bulk of insoluble iodinated protein could have adsorbed the relatively small portion of protein representing TSH.   Pure TSH has been reported by Ciereszko (3) to be active at a dose of 1 γ of protein, which, even if the difference between the units employed is allowed for, is vastly

TABLE VIII

Effect of Thyroxine on Activity of Thyrotropic Hormone Extract

Assay No.	Agents per chick	Thyroid weight	Thyroid I	Average mean cell height	Testes weight
		mg.	γ *per mg.*	μ	*mg.*
329	0	2.8	0.88	3.99	9.0
330	60 γ thyroxine	3.1	1.48	3.98	9.0
331	60 " " + 2 units TSH	5.2	0.12	8.21	16.8
332	2 units TSH	7.0	0.07	8.29	18.5

TABLE IX

Simultaneous Effect of Iodine on Thyrotropic and Gonadotropic Activity

Assay No.	Agents per chick	Thyroid weight	Testes weight
		mg.	*mg.*
195	None	3.19	9.20
196	2 units iodo complex	3.25	12.00
212	2 " untreated	6.60	16.40
199	20 " iodo complex	4.90	26.50
200	20 " untreated	9.60	26.30

purer than the preparation used in these experiments (1 unit = 400 γ of protein).   Consequently TSH might not have been affected by iodine, and the observed loss of activity might be ascribed to adsorption of TSH on the remaining 399 parts of insoluble iodinated protein.   This is quite unlikely, however, because it has been observed that the chick is able to liberate TSH from strong adsorbents.

Another possible explanation for the abolition of TSH activity by iodine concerns the bioassay, since the iodinated extract, being insoluble, might not be sufficiently absorbed from the tissues to exert a thyrotropic effect. This is unlikely because the bioassay extends over a 3 day period and because it is doubtful that any change in absorption rate, either accelerated

or depressed, could account for a complete loss of effect of a maximal dose of hormone assayed in the manner described. Experimental evidence (Table IX) is available showing that altered tissue absorption is not a major factor in the bioassay method. This evidence was obtained by simultaneously assaying in the same chicks the gonadotropic and thyrotropic activity of the extract before and after iodination, utilizing the weight of the testes as a measure of total gonadotropic activity. The overall gonadotropic potency was not markedly altered by iodination, while there was simultaneously a complete loss of TSH activity. The observed inactivation of TSH extract, therefore, cannot be attributed to an effect of iodine on tissue absorption.

In view of this discussion, a tentative explanation is that free iodine has induced a chemical change, presumably oxidative in nature, in the thyrotropic hormone which becomes inactivated.

SUMMARY

1. The addition of free iodine to a pituitary extract resulted in abolition of 90 to 100 per cent of the thyrotropic activity, but no change in over-all gonadotropic activity.

2. The degree of inactivation of thyrotropic potency was proportional to the amount of iodine added.

3. Provided sufficient iodine was present, the inactivation of thyrotropic potency was independent of the amount of extract tested.

4. The failure of the iodine-treated pituitary extract to evoke thyrotropic activity could not be ascribed to the biological action of iodine, to the action of any thyroxine that might be formed in the reaction mixture, or to an effect on tissue absorption.

BIBLIOGRAPHY

1. Astwood, E. B., and Bissell, A., *Endocrinology,* **34,** 282 (1944).
2. Bergman, A. J., and Turner, C. W., *Endocrinology,* **24,** 656 (1939).
3. Ciereszko, L., *J. Biol. Chem.,* **160,** 585 (1945).
4. Cortell, R., and Rawson, R. W., *Endocrinology,* **35,** 488 (1944).
5. Fraenkel-Conrat, J., Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.,* **135,** 199 (1940).
6. Rawson, R. W., Graham, R. M., and Riddell, C. B., *Ann. Int. Med.,* **19,** 405 (1943).
7. Rawson, R. W., and Salter, W. T., *Endocrinology,* **27,** 155 (1941).
8. Rawson, R. W., Sterne, G. D., and Aub, J. C., *Endocrinology,* **30,** 240 (1942).
9. Smelser, G. K., *Endocrinology,* **23,** 429 (1938).
10. Stimmel, B. F., and McCullagh, D. R., *J. Biol. Chem.,* **116,** 21 (1936).

THE CONJUGATED, NON-PROTEIN, AMINO ACIDS OF PLASMA

III. PEPTIDEMIA AND HYPERPEPTIDURIA AS A RESULT OF THE INTRAVENOUS ADMINISTRATION OF PARTIALLY HYDROLYZED CASEIN (AMIGEN)

By HALVOR N. CHRISTENSEN, ELEANOR L. LYNCH, AND JOHN H. POWERS

(*From The Mary Imogene Bassett Hospital, Cooperstown, New York*)

Almost no information was available concerning the fate of peptides introduced intravenously prior to the observation that the peptide-rich hydrolysate of casein, "amigen," could be infused without unfavorable results. Methods previously used for the demonstration of peptides in plasma are invalid and such substances may be absent from normal plasma (1). Substances with the properties of peptides have long been known to occur in urine (2). Madden *et al.* (3) have reported that amigen administered parenterally to dogs provided far better nitrogen retention and plasma protein regeneration than acid hydrolysates of casein fortified with tryptophane. To explain the superiority of amigen White and Sayers (4) have suggested that peptides might be better utilized by tissues or more completely retained by the kidneys than are free amino acids. Acid hydrolysates of casein containing two-thirds of the amino acids in the bound form administered intravenously to dogs have maintained nitrogen balance (5). We have examined the metabolism of the peptides of amigen by observing their concentrations in plasma and urine following infusion of the hydrolysate. The combination of chemical deproteinization (to eliminate proteolytic activity) and dialysis through cellophane (to complete the elimination of protein) was recommended by our findings for the least equivocal demonstration of the presence in tissues of conjugated, non-protein, amino acids (as in peptides).

We have observed that during the infusion of 50 gm. of amigen into young men dialyzable amino acid conjugates soluble in tungstic acid (properties shown by the peptides of amigen) were present in plasma to the extent of 2.3 mg. per cent (infusion over 5 hours) to 4.9 mg. per cent (infusion requiring 103 minutes) of α-amino nitrogen (manometric ninhydrin procedure (6)) released by acid hydrolysis, and that this peptidemia persisted for 3 to 6 hours or more after the infusion (Table I). The elevation of conjugated amino acids was larger and much more prolonged than that of free amino acids, despite the fact that two-thirds of the amino acids of amigen appeared to be free. Loss of conjugated dialyzable α-amino nitrogen by way of the urine during the period of infusion plus 3 to 6 hours after

TABLE I

Plasma Amino Acid Changes Produced by Infusion of Amigen
The results are given in mg. per cent.

Experiment No.	α-Amino N	Before infusion	Mid-infusion	End of infusion	1 hr. after	2 hrs. after	3 hrs. after	6 hrs. after
52	Free	4.55	8.51	9.33	4.95		4.15	
	Bound diffusible*	0.08	3.83	4.86	2.11		1.09	
62	Free	3.98	7.58	8.22		3.56		3.78
	Bound diffusible	−0.04	3.32	4.66		1.19		0.34
46	Free	3.13	4.27	4.77	3.77	3.29		
	Bound diffusible	0.29	2.21	2.33	1.35	0.80		
74	Free	4.65		5.64†			3.29	3.27
	Bound diffusible	0.19		2.58†			0.98	0.21

Experiment 52, normal person, infusion during 103 minutes.

Experiment 62, patient, 26 days after fracture of pelvis with rupture of the urethra; infusion required 90 minutes.

Experiment 46, patient, 48 hours after hernioplasty; infusion during 5 hours.

Experiment 74, patient, 29 days after bilateral dissection of inguinal and femoral lymph nodes and amputation of carcinomatous penis.   Only 27 gm. of amigen infused during 50 minutes.

* Total diffusible α-amino N minus free α-amino N.

† 10 minutes after end of infusion.

TABLE II

Urine Amino Acid Changes Produced by Infusion of Amigen

Experiment No.*	α-Amino N	Pre-test period, fasting, 12 hrs.	Infusion period + succeeding 3 to 6 hrs	Extra loss	
				Net	Category in amigen infused
		mg. per hr.	mg. per hr.	mg.	per cent
52	Free	5.16	37.9	155	5
	Bound diffusible†	8.29	179	850	53
62	Free	4.03	23.8	184	6
	Bound diffusible	6.86	110	798	50
46	Free	6.42	20.8	120	4
	Bound diffusible	9.18	80.8	596	37
74	Free	5.32	11.24	41.4	2.4
	Bound diffusible	8.70	53.0	310	36

* See the notes below Table I.

† Total diffusible α-amino N minus free α-amino N.

was 36 to 53 per cent of that present in the amigen, while the loss of free amino acids was 2.4 to 6 per cent (Table II).[1]

[1] Cox and Mueller (7) recently have reported similar losses by dogs receiving amigen intravenously.

These results, together with the observation that peptides were normally excreted by the kidneys apparently in the absence of measurable concentrations in the plasma, indicate limited retention of peptides by the kidneys and limited ability of the tissues to utilize the peptides present in amigen. Hence the peptides of this partial enzymatic hydrolysate do not appear, as a group, to contribute nutritional advantage to the preparation, although special nutritional value of individual peptides is of course not excluded.

EXPERIMENTAL

1 liter (in one case 540 ml.) of 5 per cent amigen solution in 5 per cent glucose was administered by a cubital vein to convalescing surgical patients and to a normal person, all young men. Venous blood samples were taken from the opposite arm, the blood treated with heparin, and the plasma separated and deproteinized at once with tungstic acid (8). Free α-amino acids were determined immediately upon 6 ml. aliquots by reaction with ninhydrin at pH 2.5 (6). The values were corrected for the effect of the urea present. 30 or 40 ml. aliquots of the tungstic acid filtrate were dialyzed in $\frac{3}{4}$ inch cellophane tubes against 60 to 80 ml. of water, accurately measured. The water content of the wet cellophane tubes before filling was estimated by weighing. The dialyses were carried out at 5° for 24 hours on a slowly rotating wheel. The largest possible aliquot of the dialysate was concentrated and hydrolyzed by acid, and analyzed for total diffusible α-amino acid nitrogen as described before (9, 1). Portions of the measured urine output were similarly dialyzed and aliquots of the dialysate analyzed for both free and total α-amino nitrogen. All analyses were in replicate.

Properties of Peptides of Amigen—Analyses of amigen and hydrolysates thereof by the nitrous acid and ninhydrin methods made by Cox and Mueller[2] and by us indicate that one-third of the α-amino acids is bound, and that the average peptide contains 3.4 to 3.5 α-amino acid molecules. Both the free and combined amino acids of amigen added to plasma could be recovered in picric acid filtrates and in tungstic acid filtrates; *e.g.*, for tungstic acid deproteinization α-amino nitrogen was recovered as follows (mg. per cent):

	Free	Total
Unmodified plasma	3.74	4.13
Calculated, amigen + plasma	9.56	13.43
Found, amigen + plasma	9.51	13.34

The latter deproteinizing agent was selected as the more advantageous. The peptides of amigen diffused readily through cellophane.

[2] Personal communication.

SUMMARY

During the intravenous infusion of a partial enzymatic hydrolysate of casein (amigen) into young men, conjugated α-amino acids diffusible through cellophane were present in plasma in concentrations of 2.3 to 4.9 mg. per cent of nitrogen. This peptidemia exceeded the rise in free amino acids and persisted for 3 to 6 hours or more after the infusions. Loss of conjugated dialyzable α-amino acids in the urine during the period of infusion and a few succeeding hours was 36 to 53 per cent of that present in the material administered, while the loss of free amino acids was 2.4 to 6 per cent. Hence the peptides of amigen as a group appear to be less readily used by the tissues and more poorly retained by the kidneys than are the free amino acids.

BIBLIOGRAPHY

1. Christensen, H. N., and Lynch, E. L., *J. Biol. Chem.*, **166**, 87 (1946).
2. Henriques, V., and Sørensen, S. P. L., *Z. physiol. Chem.*, **63**, 27 (1909).
3. Madden, S. C., Woods, R. R., Shull, F. W., and Whipple, G. H., *J. Exp. Med.*, **79**, 607 (1944).
4. White, A., and Sayers, M. A., *J. Biol. Chem.*, **161**, 485 (1945).
5. Risser, W. C., Schenck, J. R., and Frost, D. V., *Science*, **103**, 362 (1946).
6. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, 231 (1943). Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).
7. Cox, W. M., Jr., and Mueller, A. J., *J. Nutr.*, **31**, 581 (1946).
8. Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **79**, 739 (1928).
9. Christensen, H. N., and Lynch, E. L., *J. Biol. Chem.*, **163**, 741 (1946).

CYTOCHROME *c* IN REGENERATING RAT LIVER AND ITS RELATION TO OTHER PIGMENTS*

By MARYLIZABETH W. CRANDALL AND DAVID L. DRABKIN

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*)

The development of satisfactory methods for the determination of cyto-chrome *c* in small amounts of tissue (1–3) has led to the accumulation of data upon the distribution and concentration of this important biocatalyst in various tissues of different species (1–7). Except for such information, nothing is known concerning the metabolism of cytochrome *c*. We re-garded it desirable first to gain insight into the rate of production of this pig-ment. A quantitative "depletion technique" of the type used so success-fully in Whipple's laboratory (8) for the study of hemoglobin production is not available for a constituent of practically all aerobic cells. As an approach in this direction we have applied the technique of partial hepatec-tomy and have secured data permitting the calculation of the rate of ap-pearance of new cytochrome *c* in the regenerating liver.

The early literature upon the regeneration (restoration) of the liver has been reviewed by Fishback (9). Ponfick (10) appears to be the first to have studied liver restoration after partial hepatectomy. Interest in this experi-mental technique has been revived by the more modern, careful studies of Higgins (11), Brues (12, 13), and Marshak (14) and their collaborators. These investigators were concerned largely with the cytological features of the restoration process, which has been established (12, 13) as true hyper-plasia (production of new tissue) in contrast to hypertrophy (enlargement of existing tissue).

Biochemical investigations of gaseous exchange as a measure of rate of glycolysis (15), riboflavin content (16), and content of enzymes such as catalase, arginase (17), and cytochrome oxidase (18) have resulted in the conclusion that regenerating or restored liver, following partial hepatec-tomy, and adult normal liver appear to be chemically indistinguishable. In contrast, embryonic and neoplastic livers have a high anaerobic glycoly-sis (15) and are low in certain essential biocatalysts (16–18). However, in none of this chemical work has attention been directed towards the quan-

titative aspects of the restoration process. The analytical data cannot be used to evaluate the rate of production, or other metabolic features, of the components measured. Approaches to the use of partial hepatectomy as a metabolic technique have been made in studies of vitamin A (19) and plasma proteins (20). The work on vitamin A, a very readily mobilizable component of liver (19), has been limited to analyses in a very short post-operative period (3 to 4 hours), and therefore cannot be related unequivo-cally to the restoration process. The investigation of Chanutin and col-leagues (20) was confined to the effect of partial liver excision upon the plasma proteins, presumably with no simultaneous study of changes in the proteins or other constituents of the liver during restoration.

In applying the partial hepatectomy technique to the study of the metab-olism of cellular components in the intact animal, it is essential that the experimental procedure be shown to be quantitatively reproducible. Data will be presented from albino rats of our colony fully confirming earlier demonstrations (11–13, 15) of the relative constancy of relationship in this species between liver mass and body mass, and particularly between ex-cised lobes, remaining tissue, and total liver. This constancy of anatomical relationships in liver, which now appears established in the rat, supplies the necessary base-line for quantitative determinations. The surgical excision of rat liver tissue is simple and rapid. Owing to the distinctive multilobed character of the liver in this species (Fig. 1), the operative procedure may be aptly designated as *liver lobectomy*. The whole metabolic procedure is economical of time, since total regeneration (restoration) after removal of two-thirds of the liver occurs within 10 to 14 days in adult rats. Thus, the measurement of the restoration of tissue components in the regenerating liver of the rat as a metabolic technique has the desirable attributes of re-producibility, ease of application, and economy of time.

During the course of this work sufficient additional data upon the cyto-chrome *c*, myoglobin, and hemoglobin concentrations have been gathered to permit a reliable estimate and comparison of the total content in the body of the rat of these three main hemin derivatives. Such data are of interest from the standpoint of the respective metabolic rôles of the substances under consideration.

Experimental Methods

Analytical Procedures—The concentration of cytochrome *c* in individual rat organs was determined by the direct micro spectrophotometric method of Rosenthal and Drabkin (3).[1] The total cytochrome *c* of the body (Table

[1] We again use the molecular weight of 13,000, corresponding to Theorell's value of 0.43 per cent for the iron content of pure cytochrome *c* (21). From their recently pub-lished data we conclude that Keilin and Hartree (22) have demonstrated that the bio-catalytic activities of cytochrome *c* preparations with 0.34 and 0.43 per cent iron are

II) was obtained by a summation of values calculated from the analytical data for the individual organs of a representative 250 gm. male rat. The organ weights are approximations based upon three rats of this size from our colony.

The myoglobin was extracted by a quantitative volumetric application (with appropriate use of dilution factors) of Morgan's modification of Theorell's method (23). The isolation was carried to the stage of separation of hemoglobin and solution of the muscle pigment in 3 M phosphate buffer (Morgan (23)). The quantitative determination of the myoglobin in this solution was by either of two procedures. (1) The pigment was totally converted to cyanmetmyoglobin[2] by the addition of ferricyanide and cyanide, and the concentration determined spectrophotometrically, with the constant ϵ ($c = 1$ mM per liter, referred to a molecular weight of 16,450, based on 0.34 per cent of iron, $d = 1$ cm.) $= 11.3$ at wave-length 540 mμ.[3] This procedure was the basis for our derivation of the total myoglobin content of the rat organism (Table III). (2) An alternative microprocedure, applicable to as little as 1 gm. of muscle relatively rich in myoglobin, or to 3 to 8 gm. of myoglobin-poor muscle (as in the present work on the rat), has also been used (Table III). In this method the myoglobin in the 3 M phosphate buffer (extracted as in Procedure 1) is transferred into aqueous, strongly alkaline pyridine. The pyridine layer separates by stratification, and the myoglobin is determined spectrophotometrically after the addition of solid dithionite ($Na_2S_2O_4$) as pyridine ferroprotoporphyrin (reduced pyridine hemochromogen), with ϵ ($c = 1$ mM per liter, $d = 1$ cm.) $= 29.1$ at wave-length 557 mμ (25) as a provisional constant. The analysis is carried out with our 3.4 cm. capillary cuvette-diaphragm technique (3). A more detailed account of these methods for myoglobin, developed by one of us (D. L. D.), will be presented in a later communication.

The hemoglobin concentration was determined spectrophotometrically as cyanmethemoglobin, with the constant ϵ ($c = 1$ mM per liter, $d = 1$ cm.)

---

proportional to their iron content (equivalent per atom of iron). We have difficulty in accepting the argument of the English investigators that their data suggest that cytochrome c with 0.43 per cent of iron (mol. wt. 13,000) is a degradation product (rather than a purification) of the "natural" cytochrome c with 0.34 per cent of iron (mol. wt. 16,450). Keilin and Hartree (22) appear rather to have established the high biological activity of the lower molecular weight cytochrome c.

[2] An analogue of cyanmethemoglobin, with practically identical spectroscopic properties (24).

[3] In the notation ϵ ($c = 1$ mM per liter, $d = 1$ cm.), $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration, c, is expressed in mM per liter, the depth, d, in cm., the intensity of incident radiation, I_0, is 1.0, and the intensity of transmitted radiation, I, is expressed as a fraction of unity. The value of the constant for the maximum of cyanmetmyoglobin has been established upon an iron basis by one of us in other work (24), details of which will be reported separately.

= 11.5 at wave-length 540 mμ (26). The total hemoglobin of the animal (Table III) was calculated from (gm. of hemoglobin per 100 ml./100) $\times$ (plasma volume in ml.)/(1 − hematocrit, written as a fraction of unity). The values for the concentration of hemoglobin and hematocrit were in good agreement with earlier extensive determinations of these quantities in the rat in our laboratory (27). The value of plasma volume is based upon a single determination with the dye T1824 (28), and should be regarded as an approximation sufficient for our present purpose.
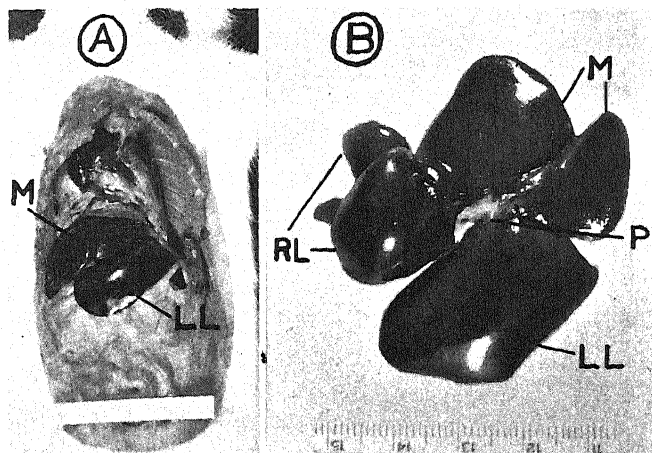
Fig. 1. Structural relationships of rat liver. A, liver *in situ*. B, whole liver excised, with notched median lobe turned back. LL, left lateral lobe, which presents first at the partial hepatectomy operation; M, median lobe; RL, right lateral and caudal lobes; P, pedicles of the left lateral and median lobes, to which ligatures are applied for excision of either the left lateral lobe alone or the left lateral plus the median lobe. The linear dimensions are indicated by the cm. scales.

Partial Hepatectomy (Liver Lobectomy)—Adult albino rats of 184 to 332 gm. in weight (averaging 150 to 200 days old) were employed. The animals of our laboratory colony are originally of Wistar Institute stock, inbred for many generations (22 years) under the supervision of Dr. J. H. Jones. The rats were fasted preoperatively for 18 to 24 hours. The surgical technique was simple, but complete asepsis was observed. After moderate ether anesthesia, enough to produce complete relaxation, the abdomen from the sternal region down was shaved and sterilized. The abdomen was then opened by means of a longitudinal, mid-line incision from over the xiphoid process caudally for about 3 to 4 cm. The desired left lateral or left lateral and median lobes were readily delivered. These lobes are on separate pedicles (P, Fig. 1) to which a hemostat was applied. A silk ligature was firmly tied around one or both pedicles beyond the hemostat, depending on whether the left lateral lobe alone (in most of our ex-

periments) or the left lateral and median lobes were to be removed. By division of the pedicle, the lobe or lobes were then excised and transferred to a small air-tight vessel (such as a weighing bottle), previously provided on its bottom with a piece of filter paper dampened with 0.9 per cent NaCl. (This provision is to prevent dehydration by evaporation before weighing the excised tissue.) The anesthesia was now usually stopped, and the abdomen closed by silk sutures, the peritoneum and muscle layer with a baseball stitch, and the skin layer separately with interrupted sutures about $\frac{1}{4}$ inch apart. The incision area was coated lightly with Whitehead's varnish. For the 1st postoperative day the animals were kept in a warm room (temperature about 27°). The whole surgical procedure took no more than 10 minutes and was free from mortality. However, some caution is advisable. Rat liver is rather friable, and must be handled gently. Also, care must be taken to avoid including a portion of the remaining right lateral lobe in the ligation. Bandages or adhesive tape should not be used during wound healing.

Since the terminology for the lobes of rat liver is not consistent, we follow that used by Higgins and Anderson (11). Fig. 1, A and B, is given for orientation.

As quickly as possible after the operation the excised liver lobe or lobes were rinsed with water, adhering fluid was blotted off with filter paper, and the tissue accurately weighed. The determinations of cytochrome c, dry weight, and protein-bound phosphorus were then carried out on accurately weighed aliquots. Dry weight was obtained by heating 50 to 60 mg. of minced tissue in micro crucibles for 2 hours at 100°, followed by cooling in a desiccator over $CaCl_2$. Protein-bound phosphorus as an index of cellularity was determined as described by Rosenthal and Drabkin (6).

After a period of 14 days, the interval of time which has been found (11, 12) adequate for complete restoration of the liver, the rats were sacrificed by stunning, followed immediately by decapitation. This procedure afforded moderately good exsanguination. The entire restored liver was removed, treated as above, and accurately weighed. Analytical data were secured not only on the regenerated liver, but also on the heart, kidney cortex, and skeletal muscle of each animal. During 13 days of the restoration process the rats were maintained either on a modified Steenbock stock diet (29),[4] adequate in protein, or on an enriched protein diet, the stock diet bolstered with casein.[4] The animals were then fasted for 1 day before the termination of the experiment. This was deemed necessary to assure com-

[4] Stock diet, ground yellow corn 74, linseed oil meal 16, crude casein 5, alfalfa 2, NaCl 0.5, $CaCO_3$ 0.5, and yeast 2 parts per hundred. This diet affords approximately 20 per cent of protein (personal communication from Dr. J. H. Jones). The enriched protein diet was the stock diet 80 parts plus casein (80 per cent protein) 20 parts. This mixture afforded approximately 32 per cent of protein.

TABLE I

Concentration (C) of Cytochrome c in Micrograms per Gm. of Wet Weight of Tissue in Control Rats, Fasted 18 to 24 Hours

Rat No.	Sex	Body weight	Liver		Heart		Kidney cortex		Skeletal muscle	
			C	$W:D^*$	C	$W:D^*$	C	$W:D^*$	C	$W:D^*$
		gm.								
1	M.	223					102		61	3.79
2	F.	195			399	3.95	342	4.10	55	3.98
3	M.	179			528	4.45	413	3.56	93	3.90
4	"	272			600	4.30	337	3.67	86	3.78
5	"	437			414	4.68	106	4.39	97	3.84
6	"	362			443	4.55	349	4.10	93	4.15
7	"	361			206	4.24	181	3.82	64	3.89
8	"	384			508	4.05	390	3.95	60	4.01
9	"	415			377	4.18	460	3.82	128	3.78
10	"	283			434	4.45	408	3.97	113	3.76
11	"	334			455	4.36	385	4.23	83	3.81
12	"	227			494	4.52	309	3.97	131	3.94
13	"	291			495		430		137	3.76
14	"	231			490	4.19	533	4.02		3.78
15					574	4.12	259	4.16	134	
16	M.	328			478	4.24		3.76	125	3.90
17	"	232	296	3.03	429	4.08	319	4.17	135	3.82
18	"	300			397	4.27	490	4.17	147	4.05
19	F.	192	164	4.40	425	4.76	431		114	4.38
20	M.	314	193	3.15	498	4.01	342	4.13	56	
21	"	306	273	3.36	478		400	4.55	48	3.44
22	"	278	285	3.28	348	4.20	369	4.27	106	3.87
23	"	306	257	3.18	312	4.82		3.98	98	3.89
24	"	292	329	3.33	474	4.66	347	4.42	114	4.04
25	"	297	260	3.07	467	4.26	358	4.00		3.85
26	"	357	334	3.04	440	4.45	407	4.29	84	3.89
H1†	F.	184	96	3.77						
H2†	"	190	159	3.46						
H3†	"	198	131	3.38						
H4†	"	201	105	3.34						
H5†	M.	267	218	3.68						
H6†	"	268	180							
H7†	"	221	181	4.01						
H8†	"	288	268	3.43						
H9†	"	332	290	3.37						
Mean ± s.e.‡......			223	3.43	447	4.34	352	4.07	98	3.89
			±18	±0.09	±16	±0.05	±21	±0.05	±6	±0.04

* $W:D$ = wet weight to dry weight ratio of tissue.

† Rats H1 to H9 are the partially hepatectomized series. These analyses are on the liver tissue excised at operation.

‡ Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

parable conditions of alimentation (which affects the composition of "storage" tissues like liver (30)) in the pre- and postregeneration periods.

Results

Our data upon the cytochrome c concentration of liver, heart, kidney cortex, and skeletal muscle of thirty-five "control" fasted rats are collected in Table I. The values for cytochrome c in liver lobes excised ("internal controls") in the partially hepatectomized series (Rats H1 to H9) are included. No difference in the cytochrome c content is apparent from the values obtained in liver from these animals and from non-operated, normal fasted rats (compare the analyses of Rats H1 to H9 with Rats 17 to 26). Similar wet weight to dry weight ratios, $W:D$, are also apparent in these two groups of animals. While it is convenient in the present work to express the cytochrome c concentrations on a wet weight basis, the $W:D$ ratios are supplied as an index of the water content of tissues, which is evidently quite constant in our series of analyses.

The present values for cytochrome c agree well with those previously reported from our laboratory (3, 6) for rat liver, heart, and kidney cortex. The results for liver not only confirm the earlier high values, but indeed are somewhat higher as well as more consistent. In these fasted animals, in contrast to those previously used (3, 6), solutions of isolated cytochrome c relatively free from turbidity were regularly obtained. This finding appears to support the conclusion of Rosenthal and Drabkin (3) that lower values for the pigment and presence of turbidity are in part attributable to the content of glycogen. As before, while analyses for the cytochrome c of rat heart and kidney cortex are of the same magnitude as those found by other investigators (1, 2), our results for liver are markedly higher. One of us (D. L. D.) has examined a number of rat liver extracts, prepared according to Rosenthal and Drabkin (3), in which simultaneous analyses for cytochrome c were carried out by a slightly modified (for adjustment to larger quantities) Potter-DuBois kidney succinic dehydrogenase technique (2) and by our direct micro spectrophotometric method, $Na_2S_2O_4$ being employed for reduction (3). Similar results were yielded by the independent analytical procedures. Hence our values for cytochrome c in liver, significantly higher than those reported by the Wisconsin workers (2), cannot be ascribed to differences in the spectrophotometric steps of the analytical methods.

In Tables II and III, which are self-explanatory, the data required for the estimation of the total cytochrome c, myoglobin, and hemoglobin of the rat (reference body weight 250 gm.) are recorded. The quantities in descending order of magnitude are hemoglobin 3190 mg., myoglobin 101 mg., and cytochrome c 14.4 mg. The ratio of hemoglobin to myoglobin to cytochrome c is hence 222:7:1 in this species. It is of interest that, while

the concentration of cytochrome c is high in rat tissues (Rosenthal and Drabkin (6)), the concentration of myoglobin appears to be quite low in comparison with other species (dog, Whipple (31)). Whether any special

TABLE II

Total Cytochrome c Content of Young, Adult, Male Albino Rat, Fasted 18 to 24 Hours

The values are calculated from analytical data for a reference body weight of 250 gm.

Organ	Weight of organ*	Per cent of body weight	Cytochrome c	
			Per gm. tissue, wet weight	Total in organ
	gm.		γ	γ
Skeletal muscles........................	113.0	45.2	98†	11,080
Liver.................................	6.50	2.6	223†	1,450
Kidneys..............................	1.63	0.65	352†	573
Abdominal organs‡......................	20.8	8.3	18§	374
Skin.................................	46.5	18.6	8§	372
Heart................................	0.73	0.29	447†	326
Brain................................	1.93	0.77	82‖	158
Blood................................	20.2¶	8.1	1.3§	26
Testes...............................	2.50	1.0	10.2§	26
Lungs................................	1.42	0.57	5.2‖	7
Skeleton.............................	26.3	10.5	**	
Rest.................................	8.49††	3.4	**	
Total..............................	250	100		14,392

* Except for the blood, based upon measurements of three male fasted rats of approximately 250 gm. each.

† Mean values of analyses reported in Table I.

‡ Exclusive of liver and kidneys.

§ Data on aliquots from pooled tissue specimens of the rats used for the determination of organ weights.

‖ Data of Rosenthal and Drabkin (6).

¶ Calculated from a plasma volume determined with the dye T1824 (28) in one animal = 9.98 ml., hematocrit = 48 per cent, and specific gravity = 1.05.

** Cytochrome c concentration not determined; assumed to be negligible in these tissues.

†† The difference between the total body mass of 250 gm. and the summation of the other components.

significance may be attached to this apparent reciprocal relationship of cytochrome c and myoglobin in the rat must await data on these relationships now being gathered for other species (dog, horse, and man) in our laboratory. Although the cytochrome c concentration (Table I) is highest in rat heart and kidney, the liver and muscles (Table II) contain nearly 90

per cent of the total cytochrome c (12.53 out of 14.39 mg.), and the muscles alone, with a relatively low concentration of the biocatalyst, contain, because of their large share of the total mass of the organism, nearly 80 per cent.

Data upon the concentration of cytochrome c in excised liver and in the restored liver of nine partially hepatectomized rats are presented in Table IV. Supplementary data are also given for heart, kidney cortex, and muscle of these animals. It is evident that the concentrations of cytochrome c in the various tissues, including liver, of rats subjected to the ac-

TABLE III

Total Myoglobin and Hemoglobin Contents of Young, Adult, Male Albino Rat of 250 Gm. Body Mass

Organ	Weight of organ*	Volume of organ	Myoglobin		Hemoglobin	
			Per gm. tissue,† wet weight	Total in organ	Per 100 ml. tissue	Total in organ
	gm.	*ml.*	*mg.*	*mg.*	*gm.*	*gm.*
Skeletal muscles...............	113.0		0.89	100.6		
Heart........................	0.73		0.91	0.66		
Blood........................	20.2	19.2‡			16.6	3.19§
Total in body				101		3.19

* See Table II.

† Analyses by Procedure 1 (see "Experimental methods") on 80 and 6.02 gm. of pooled skeletal and cardiac muscle respectively from six rats. Individual analyses on muscle of hind limb from Rats 8, 9, and 15 (Table I) by the micro spectrophotometric technique (Procedure 2, "Experimental methods") were respectively 0.50, 0.68, and 0.57 (mean = 0.58) mg. per gm. of wet weight of tissue.

‡ Calculated from plasma volume 9.98 ml. and hematocrit 48 per cent (Table II).

§ For calculation see "Experimental methods."

tive hyperplastic process in liver, following its partial excision, were similar in magnitude to corresponding values in the controls (Table IV, n. values for liver, and Table I for the other tissues). The sex had no influence on the results. The restored liver had a slightly, but significantly, lower water content ($W:D$ ratios, Column 5) than that of corresponding excised (control) liver tissue. While the difference in the means of the $W:D$ values for excised and restored liver is only -0.30 ($3.55 - 3.25$), this difference is statistically significant. Tested by Fisher's method (32), the individual differences in $W:D$ ratios yield a value of $t = 0.30/0.072$ (the standard error of mean differences) $= 4.16$. This value corresponds in Fisher's table of t (32) to a probability, P, of less than 0.01 that the difference in the means

is a matter of chance. Deviating in the opposite direction from the water content was the protein-bound phosphorus (Column 6, Table IV), which

TABLE IV

Concentration (C) of Cytochrome c in Liver and Other Tissues of Rats Subjected to Partial Hepatectomy

Rat No.* (1)	Sex (2)	Body weight (3)	Liver			Heart		Kidney cortex		Skeletal muscle	
			C, wet weight (4)	W:D† (5)	P,‡wet weight (6)	C, wet weight (7)	W:D (8)	C, wet weight (9)	W:D (10)	C, wet weight (11)	W:D (12)
		gm.	γ per gm.		γ per gm.	γ per gm.		γ per gm.		γ per gm.	
H1 (n.)	F.	184	96	3.77	921						
H1 (r.)		196	187	3.48	950	713	4.74	294	3.79	124	3.98
H2 (n.)	"	190	159	3.46	839						
H2 (r.)		187	214	3.15	1020	468	4.48	293	4.05	83	3.99
H3 (n.)	"	198	131	3.38	1005						
H3 (r.)		191	154	3.01	1130	360	3.98	228	4.40	101	4.00
H4 (n.)	"	201	105	3.34	704						
H4 (r.)		201	191	3.14	1130	496		309	3.65	119	4.03
H5 (n.)	M.	267	(218)	3.68	1012						
H5 (r.)§		273	(174)		1002	381	4.20	371	4.01	125	3.99
H6 (n.)	"	268	180		891						
H6 (r.)§		281	184	3.36	1090		4.21	403	4.37	113	4.18
H7 (n.)	"	221	181	4.01	1020						
H7 (r.)§		247	203	3.33	979	392	4.96	375	4.23	90	4.07
H8 (n.)	"	288	268	3.43	1060						
H8 (r.)		275	265	3.22	1120	385	4.58	457	4.24	117	3.99
H9 (n.)	"	332	290	3.37	981						
H9 (r.)		321	289	3.30	1090	394	4.22	386	3.94	121	4.05
Mean ± s.e.‖ (n.)		239	176 ±25	3.55 ±0.06	937 ±37						
Mean ± s.e. (r.)		241	211 ±16	3.25 ±0.05	1057 ±23	449 ±41	4.42 ±0.12	346 ±23	4.08 ±0.09	110 ±5	4.03 ±0.02

* The letters in parenthesis, (n.) and (r.), designate respectively the analyses on the excised liver ("internal controls"), and on the tissues of the same rats 14 days postoperatively (full restoration of liver).

† $W:D$ = wet weight to dry weight ratios.

‡ P = protein-bound phosphorus (6).

§ Rats H5, H6, and H7 were on the enriched protein diet; the other rats were on the stock diet (see foot-note 4).

‖ Standard error = $\sqrt{\Sigma d^2/n(n-1)}$. The values (in parentheses) for cytochrome c in the liver of Rat H5 were excluded in calculating the means, since the analysis for cytochrome c in the restored liver of this animal (r. value) is regarded to be faulty (see the text).

was in seven out of nine rats somewhat higher in the restored liver. The difference in the mean values for protein-bound phosphorus of restored and

excised liver was +120 (1057 − 937). Tested by Fisher's method, this difference was found to be within the border line of statistical significance, with $t = 120/47 = 2.56$, and $P =$ less than 0.05. Hence it must be concluded from the protein-bound phosphorus analyses that the restored liver, even after 14 days of regeneration, had a slightly higher cellularity (6) than the original liver tissue. It may be noted that the cytochrome c concentration (Column 4) shows a change in a direction similar to that of the protein-bound phosphorus. In only one animal, Rat H5, is the value for cytochrome c concentration in the restored liver appreciably lower than in the excised tissue. This result is probably analytically erroneous. When the results on this rat are excluded, the difference in means for cytochrome c in restored and excised liver is +35 (211 − 176) and is significant, according to Fisher's criterion ($t = 2.59$, $P =$ less than 0.05). It also appears significant that the mean values for cytochrome c concentration per microgram of protein-bound phosphorus are similar in restored and excised liver (211/1057 = 0.200, and 176/937 = 0.188 γ respectively).

The following values with their standard errors illustrate the constancy of structural relationships of the liver in the young adult rats of our colony. In four fed females, weight of individuals from 254 to 283 gm., the total liver = 3.13 ± 0.10 per cent of the body weight, while the left lateral lobe = 34.4 ± 0.7, the median lobe = 33.7 ± 0.8, the right lateral and caudal lobes = 31.9 ± 0.9, and the left lateral plus the median lobe = 68.1 ± 0.9 per cent of the liver mass. In animals fasted 18 to 24 hours (ten males and two females), weight of individuals from 182 to 357 gm., the total liver = 2.62 ± 0.04 per cent of the body weight, while the left lateral lobe = 36.2 ± 0.2, the median lobe = 32.2 ± 0.2, the right lateral and caudal lobes = 31.6 ± 0.2, and the left lateral plus the median lobe = 68.4 ± 0.2 per cent of the liver weight. It may be noted that fasting for 18 to 24 hours markedly altered the percentage liver of body mass, and the standard errors suggest that appreciably closer agreement of values was obtained in the fasted animals. This is not surprising, since variations are recognized to exist in storage substances of growing animals fed *ad libitum*.[5] In fasted rats our value for the percentage mass of the left lateral plus the median lobe is in excellent agreement with that reported by Brues *et al.* (12), who concluded that the relationship of total liver to individual lobes is a safer base-line to employ in the interpretation of the quantity of regeneration than is the ratio of body mass to liver mass.

In Table V calculations, based on the data in Table IV and those given above in the text, are recorded for the quantity of new cytochrome c which has appeared in the regenerating liver in a postoperative period of 14 days.

[5] Dr. H. M. Vars has found (personal communication to us) that in similar rats fasted for 18 to 24 hours the glycogen is low (of the order of 0.5 to 0.6 per cent) and quite constant.

It is obvious from the values in Table V that from our data the same con- clusions could be derived whether the weight of the original liver was taken

TABLE V

Quantity of New Cytochrome c in Rat Liver after 14 Days of Restoration

Rat No. (1)	Body weight (initial) (2)	Amount of liver excised (3)	Weight of original liver (4)		Amount of remaining liver (5)		Cytochrome c in remaining liver‡ (6)		Weight of restored liver (7)	Total pigment§ (8)	Cytochrome c in restored liver New pigment‖ (9)			
			(a)*	(b)†	(a)	(b)	(a)	(b)			(a)		(b)	
	gm.	gm.	gm.	gm.	gm.	gm.	γ	γ	gm.	γ	γ	per cent	γ	per cent
H1	184	2.47	4.82	3.61	2.35	1.14	226	109	6.01	1123	897	79.8	1014	90.2
H2	190	3.01	4.98	4.40	1.97	1.39	313	221	4.01	858	545	63.5	637	74.2
H3	198	1.76	5.19	4.86	3.43	3.10	449	406	3.61	556	107	19.2	150	27.0
H4	201	1.52	5.27	4.20	3.78	2.68	397	282	5.38	1028	631	61.4	746	72.6
H5	267	2.65	7.00	7.32	4.35	4.67	949	1020	6.14	1069	120	11.2	49	4.6
H6	268	2.94	7.03	8.12	4.09	5.18	737	933	6.93	1275	538	42.2	342	26.8
H7	221	2.42	5.79	6.68	3.37	4.26	610	771	5.65	1147	537	46.8	376	32.8
H8	288	2.84	7.55	7.57	4.71	4.73	1262	1268	5.99	1588	326	20.5	320	20.2
H9	332	3.11	8.70	8.59	5.59	5.48	1621	1590	7.51	2170	549	25.3	560	25.8
Mean¶										1313	575	48.5	571	48.9
Rate, per day, of appearance of new cytochrome c											41	3.5	41	3.5

* Values in columns labeled (a) calculated on the basis of the weight of the original liver = 2.62 per cent of body weight (see the text and Column 2, this table).

† Values in columns labeled (b) calculated on the basis of percentage of liver tissue excised (from the relationship of lobes to total liver given in the text). In Rats H1 and H2 both the left lateral and the median lobes were removed, or 68.4 per cent was excised (see the text); in the remaining animals only the left lateral lobe (= 36.2 per cent) was removed.

‡ Calculated by multiplying the values for concentration of cytochrome c in excised liver (n. values in Table IV, Column 4) by the corresponding weights of remaining liver tissue (Column 5, this table).

§ Calculated by multiplying the values for the concentration of cytochrome c in restored liver (r. values in Table IV, Column 4) by the corresponding weights of the total restored liver (Column 7, this table).

‖ Total pigment (Column 8) minus corresponding values for cytochrome c in remaining liver tissue (Column 6).

¶ Data from Rats H3 and H5 were excluded in calculating the means. In Rat H3 the amount of restoration of liver was abnormally low; in Rat H5 the analytical value for cytochrome c concentration in the restored liver is thought to be erroneous (see the text).

as a fixed percentage of body weight (columns (a)) or was based on a definite relationship of individual lobes to total liver (columns (b)). The rate of appearance of new cytochrome c in the regenerating liver was found to

average 41 γ per day. This was equivalent to a daily increment in pigment of 3.5 per cent, and was of the same magnitude (percentage basis) as the increase in new liver tissue. For unknown reasons, in one rat (No. H3) appreciable restoration of the liver failed to occur. In another animal (Rat H1) the restored liver had an appreciably larger weight than that calculated for the original liver. This phenomenon of the regeneration process has been observed previously (11).

<div align="center">DISCUSSION</div>

The technique of partial hepatectomy may serve not only for the study of the metabolism of tissue constituents (as in the present work), but also in the elucidation of cellular regeneration, a phenomenon of the utmost importance. For both purposes an obvious approach is a study of the influence of the diet. In the experiments reported here (Tables IV and V) dietary considerations were minor. No difference was observed on an adequate protein (20 per cent) and on an enriched protein (32 per cent) diet, either in the rate of regeneration or in the rate of appearance of new cytochrome c.

In describing the phenomenon of increase in total cytochrome c of liver which occurred in the process of tissue restoration, we have used the phrase *rate of appearance of new cytochrome c in liver* rather than *rate of production of cytochrome c*. We did not employ the latter description since we wished to avoid the suggestion that the present experiments have demonstrated a production *de novo* of cytochrome c in the body. It may be that cytochrome c had been manufactured from its building stones in the newly formed liver tissue. There are indirect arguments in favor of this possibility. (1) The concentration of cytochrome c and the cellularity (protein-bound phosphorus, discussed above) are proportional. (2) The concentration of the pigment is similar in restored and in normal, adult liver. This similarity of concentration in regenerated and normal liver has been found (17) to hold also for arginase, an exclusive constituent of liver. (3) No evidence exists that a protein such as cytochrome c can migrate from one tissue to another. On the other hand, cytochrome c is an unusual protein of low molecular weight (21), with a universal distribution in the cells of all aerobic tissues. While an appreciable amount (571 γ, Table V) of new cytochrome c had appeared in the liver in 14 days of restoration, and while the concentration of cytochrome c in heart, kidney, and skeletal muscle had remained unaltered following partial hepatectomy (compare the values in Tables I and IV), it may be questioned whether the new pigment in liver had not been preformed in another tissue. The only tissue from which cytochrome c could have been mobilized, without detection by our analytical procedure, is the skeletal muscle. The latter contains a total of 11,080

γ of cytochrome c (Table II). The amount of new pigment in liver is only 5.2 per cent ($(571 \times 100)/11{,}080$) of the above quantity. Special techniques will be required to settle the issue whether cytochrome c can be transferred from one tissue to another.

The rate of appearance (41 γ per day, or a daily increment of 3.5 per cent, Table V) of cytochrome c in regenerating liver is not small on a percentile basis when compared to the rate of production of hemoglobin with Whipple's depletion technique (8). Hemoglobin formation under the stimulus of constant blood withdrawal is of the order of 1 gm. per kilo per day (33). Assuming a blood volume of 8 per cent and a hemoglobin concentration of 20 gm. per 100 ml. of blood (8), the percentage of new hemoglobin formed per day in Whipple's dogs is 6.3. Our percentage of 3.5 for the daily increment in cytochrome c in regenerating liver, therefore, is not materially different in magnitude from the percentile daily increment in hemoglobin in animals kept depleted of the blood pigment. It is also probable that higher values than 3.5 per cent might have been obtained had analyses been performed in the first few days after liver excision, when the rate of regeneration is appreciably greater (12, 13, 15).

Special attention may be called to the protein-bound phosphorus index of "cellularity." Rosenthal and Drabkin (6, 34) applied this index to the determination of the concentration of cytochrome c, and found that the concentration of cytochrome c in malignant tissues deviated most markedly from that of normal tissues when the concentration of the pigment was expressed per unit of protein-bound phosphorus (6, 34). In the case of neoplastic growths increased cellularity was accompanied by decreased cytochrome c concentration. This is in striking contrast with the hyperplastic process which follows partial liver excision. Here we have found small though significant increases in cytochrome c concentration and in protein-bound phosphorus, but both quantities have the same relationship to each other in the restored and in the excised, control liver tissue. During the course of our studies it was reported by Raska (35) that a material increase in cytochrome c concentration occurred in the remaining, hypertrophied kidney after unilateral nephrectomy in dogs. However, when Raska's values are related to protein-bound phosphorus, which he also determined, the change in cytochrome c concentration does not appear significant. Thus, the changes in cellularity which accompany the processes of hyperplasia and hypertrophy seem to differ strikingly from the neoplastic process in regard to the relationship of cytochrome c and the protein-bound phosphorus. The possible use of the protein-bound phosphorus as a chemical index of malignancy appears worthy of further investigation.

phorus, and to the above department for the use of their surgical facilities. We are also grateful to Miss Ann Claire Barnett for technical help in some of the determinations of myoglobin.

SUMMARY

Partial hepatectomy in the rat has been suggested as a useful, reproducible technique in the investigation of the metabolism of tissue constituents.

By the above means the cytochrome c in the regenerating liver has been studied. In a postoperative period of 14 days, allowing complete restoration of tissue, the total increase in new cytochrome c was 571 γ, or a daily increment of 41 γ. The rate of appearance of cytochrome c in the regenerating liver was 3.5 per cent per day. This rate compares favorably on a percentile basis with that of the production of hemoglobin in dogs depleted by hemorrhage (8, 33).

In the appraisal of the results in liver supplementary data were obtained on the cytochrome c and water contents of other tissues, heart, kidney cortex, and skeletal muscle.

The protein-bound phosphorus as an index of cellularity (6) was determined in the excised and restored liver tissue, and its value in the interpretation of the findings has been discussed.

Sufficient analytical data have been accumulated to permit a reliable estimation of the total cytochrome c, myoglobin, and hemoglobin of a young, adult albino rat (reference body weight 250 gm.). The quantities in descending order were hemoglobin 3190 mg., myoglobin 101 mg., and cytochrome c 14.4 mg. The ratio of these main hemin derivatives is therefore 222:7:1 in this species.

BIBLIOGRAPHY

1. Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
2. Potter, V. R., and DuBois, K. P., *J. Biol. Chem.*, **142**, 417 (1942).
3. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
4. Fujita, A., Hata, T., Numata, I., and Ajisaka, M., *Biochem. Z.*, **301**, 376 (1939).
5. Junowicz-Kocholaty, R., and Hogness, T. R., *J. Biol. Chem.*, **129**, 569 (1939).
6. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **150**, 131 (1943).
7. Greenstein, J. P., Werne, J., Eschenbrenner, A. B., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **5**, 55 (1944).
8. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **115**, 651 (1936).
9. Fishback, F. C., *Arch. Path.*, **7**, 955 (1929).
10. Ponfick, E., *Verhandl. deut. Ges. Chir.*, **19**, 28 (1890).
11. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, **12**, 186 (1931).
12. Brues, A. M., Drury, D. R., and Brues, M. C., *Arch. Path.*, **22**, 658 (1936).
13. Brues, A. M., and Marble, B. B., *J. Exp. Med.*, **65**, 15 (1937).
14. Marshak, A., and Walker, A. C., *Am. J. Physiol.*, **143**, 226, 235 (1945).
15. Norris, J. L., Blanchard, J., and Povolny, C., *Arch. Path.*, **34**, 208 (1942).
16. Robertson, W. von B., and Kahler, H., *J. Nat. Cancer Inst.*, **2**, 595 (1942).

17. Greenstein, J. P., Edwards, J. E., Andervant, H. B., and White, J., *J. Nat. Cancer Inst.*, **3**, 7 (1942). Greenstein, J. P., American Association for the Advancement of Science, Research conference on cancer, Washington, 192 (1945).
18. Greenstein, J. P., and Thompson, J. W., *J. Nat. Cancer Inst.*, **4**, 271 (1944).
19. Young, G., and Wald, G., *Am. J. Physiol.*, **131**, 210 (1940–41).
20. Chanutin, A., Hortenstine, J. C., Cole, W. S., and Ludewig, S., *J. Biol. Chem.*, **123**, 247 (1938).
21. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1804 (1941).
22. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
23. Morgan, V. E., *J. Biol. Chem.*, **112**, 557 (1935–36). Theorell, H., *Biochem. Z.*, **252**, 1 (1932); **268**, 46 (1934).
24. Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).
25. Drabkin, D. L., *J. Biol. Chem.*, **146**, 605 (1942).
26. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 51 (1935–36).
27. Drabkin, D. L., and Fitz-Hugh, T., Jr., *Am. J. Physiol.*, **108**, 61 (1934).
28. Gregersen, M. I., Gibson, J. J., and Stead, E. A., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **113**, 54 (1935). Gibson, J. G., 2nd, and Evans, W. A., Jr., *J. Clin. Invest.*, **16**, 301 (1937).
29. Steenbock, H., *Science*, **58**, 449 (1923).
30. McBride, J. J., Guest, M. M., and Scott, E. L., *J. Biol. Chem.*, **139**, 943 (1941).
31. Whipple, G. H., *Am. J. Physiol.*, **76**, 693 (1926).
32. Fisher, R. A., Statistical methods for research workers, London, 4th edition, 151 (1932).
33. Robscheit-Robbins, F. S., Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, **82**, 311 (1945).
34. Rosenthal, O., and Drabkin, D. L., *Cancer Res.*, **4**, 487 (1944).
35. Raska, S. B., *J. Exp. Med.*, **82**, 227 (1945).

ISOLATION AND PROPERTIES OF HYDROLECITHIN (DIPAL-MITYL LECITHIN) FROM LUNG; ITS OCCURRENCE IN THE SPHINGOMYELIN FRACTION OF ANIMAL TISSUES*

By S. J. THANNHAUSER, JOSEPH BENOTTI, and NICHOLAS F. BONCODDO

(From the Boston Dispensary, the Joseph H. Pratt Diagnostic Hospital, and Tufts College Medical School, Boston)

Sphingomyelin prepared and purified by the usual methods (1–4) was shown to be hydrolyzed enzymatically by calves' liver extracts to ceramides (lignoceryl sphingosine), phosphoric acid, and choline (5). The only fatty acid formed during the enzymatic hydrolysis was found to be palmitic acid. It was demonstrated that the fatty acid groups present in sphingomyelin in —NH—OC·R linkage (like lignoceryl sphingosine in ceramides) are not split off by the liver enzyme. The palmitic acid must therefore have originated from another source. At that time the possibility was considered that a part of the sphingomyelin contains palmitic acid in ester linkage with its free hydroxyl group. The correctness of this assumption became doubtful when all attempts to isolate a sphingomyelin palmitic acid ester were unsuccessful. In further search for the source of palmitic acid we analyzed our sphingomyelin samples for glycerol according to the micromethod of Blix (6). It was found that all samples of so called pure sphingomyelin contained considerable amounts of glycerol (7). Similar experiences were reported at the same time by Klenk and Rennkamp (8). The glycerol-containing substance could not be removed by exhaustive ether extraction or by repeated recrystallization of sphingomyelin from various solvents. Since the amount of the total phosphorus of these preparations was in agreement with that present in phosphatides, the most plausible explanation for the presence of glycerol as well as that of palmitic acid in ester linkage was the assumption that, together with the sphingomyelin, a saturated ether-insoluble monoaminophosphatide was present. This assumption was all the more appealing since Lesuk and Anderson (9) reported the isolation of a saturated, ether-insoluble lecithin from the lipide mixture of the larvae of *Cysticercus fasciolaris*, demonstrating for the first time the presence of a saturated lecithin in animal lipide mixtures. The isolation of hydrolecithin from the lipide fraction of *Cysticercus fasciolaris* larvae

proved to be a relatively simple procedure, since sphingomyelin was not found to be present.

Our efforts to isolate hydrolecithin from the sphingomyelin fractions of higher animals had to overcome very great experimental difficulties, owing to the almost identical behavior of both substances toward organic solvents and to the lack of an expedient and sensitive analytical method for their determination. Only the observation that sphingomyelin is resistant to N sodium or potassium hydroxide at 37°, in contrast to hydrolecithin which is completely hydrolyzed under those conditions, made it possible to devise a simple guiding test for the development of the preparative procedure (10). Under control of this analytical method, the ether-insoluble lipide material was repeatedly fractionated with acetone containing small amounts of glacial acetic acid. Finally a crystallized monophosphatide was obtained which was identified as dipalmitolecithin by the results of the elementary analysis and by the identification of its hydrolysis products.

The work reported in this paper was carried out with beef lung, since the composition of its ether-insoluble lipide fraction offered more favorable conditions for the isolation of the ether-insoluble phospholipides than did brain. In particular, the lipides of lung contain only small amounts of cerebrosides whose abundance in brain represents the main difficulty encountered in the fractionation and isolation of the ether-insoluble phospholipides of this organ. Lung contains much larger amounts of sphingomyelin and hydrolecithin than the other large animal organs except brain.

Hydrolecithin was also found to be present in the sphingomyelin fraction of brain and spleen. It is under investigation whether or not the hydrolecithins of brain and spleen are identical with the dipalmityl lecithin of lung.

EXPERIMENTAL

Analytical Methods for Determination of Hydrolecithin Content of Sphingomyelin Fractions

The fractionation of sphingomyelin for the isolation of hydrolecithin is accomplished by the application of the analytical micromethod of Schmidt, Benotti, Hershman, and Thannhauser (10) for the estimation of monoaminophosphatides and sphingomyelin. In this publication it is shown that monoaminophosphatides are totally hydrolyzed by mild saponification at 37°, while diaminophosphatides are stable under such conditions. Prior to the fractionation for the preparation of hydrolecithin, the lipide material is always exhaustively extracted with ether for the removal of the ether-soluble monoaminophosphatides. For this

reason the amount of acid-soluble phosphorus compounds obtained after saponification of the ether-insoluble lipide material originates exclusively from ether-insoluble monoaminophosphatides and therefore indicates the amounts of hydrolecithin present in the lipide fraction analyzed. The analytical procedure is as follows:

25 mg. of material are dissolved in 10 cc. of petroleum ether-methanol (9:1). Aliquots of 1 and 2 cc. respectively are used for the determination of the total and of the saponifiable phosphorus. Both aliquots are evaporated to dryness. The former is ashed according to the method of Fiske and Subbarow, while the latter is dissolved in 0.2 cc. of hot ethanol and emulsified in 5 cc. of N KOH at room temperature. The emulsion is shaken for 24 hours at 37°. The saponification is stopped by adding 1 cc. of 6 N HCl and 5 cc. of 10 per cent trichloroacetic acid. This procedure precipitates the sphingomyelin together with the fatty acids originating from the hydrolysis of the monophosphatides. After standing for 20 minutes, the solution is filtered. A 3 cc. aliquot of the clear filtrate is ashed and the P is determined. This phosphorus fraction (the acid-soluble P obtained after saponification) will be designated as saponifiable phosphorus in the following description. It indicates the phosphorus content of the monophosphatides present in the original lipide sample and is conveniently expressed in per cent of the total phosphorus of the original sample. Since the non-saponifiable P is identical with that of the sphingomyelin, the difference between the total and the saponifiable P represents the amount of sphingomyelin phosphorus.

Preparative Procedure for Isolation of Hydrolecithin from Lung—50 pounds of lung are minced and washed twice with acetone, filtered, and dried in a vacuum dryer at 60°. It is then ground to a powder and extracted with ether for 3 days in a large apparatus for continuous extraction. The crude ether extract is placed in the refrigerator overnight and filtered over Hyflo filter aid (Johns-Manville). The precipitated lipide is reextracted with ether in a Soxhlet extractor for several days for the purpose of removing the ether-soluble fraction (ceramides and monoaminophosphatides containing unsaturated fatty acids). The residue in the thimble is taken up in a liter of petroleum ether-methanol (9:1) and filtered. The filtrate is concentrated to a small amount of liquid of thin syrupy consistency and precipitated with a large excess of acetone (1 to 2 liters). The suspension is placed in the refrigerator overnight. The precipitate, consisting mainly of a mixture of hydrolecithin and sphingomyelin, is filtered off. It usually weighs between 20 and 40 gm. The substance contains between 3.8 and 4.0 per cent total phosphorus, of which 40 to 50 per cent is saponifiable. The mother liquor contains only negligible amounts of hydrolecithin and sphingomyelin.

This crude ether-insoluble phosphatide mixture containing hydrolecithin, sphingomyelin, and small amounts of cerebrosides is treated with 10 volumes of glacial acetic acid (300 cc. for 30 gm.), in which most of it dissolves on slight warming. The solution is allowed to stand overnight at room temperature and is then filtered. The insoluble residue is reextracted with 10 volumes of acetic acid in order to extract the remaining phosphatides. The pooled filtrates are concentrated to a very small volume and the resulting viscous material is precipitated with an excess of acetone (1000 to 1500 cc.). After standing overnight in the refrigerator, the suspension is filtered. The precipitate contains hydrolecithin and sphingomyelin in a mutual proportion of approximately 1:2, while the hydrolecithin is enriched in the filtrate in which the proportion of hydrolecithin to sphingomyelin is approximately 3:1. Further fractionation of the insoluble portion containing mostly sphingomyelin did not prove to be worth the effort, as some hydrolecithin always remained in the sphingomyelin fraction and the yield of hydrolecithin was extremely small. The acetic acid filtrate is concentrated to dryness under reduced pressure and dried over NaOH in an evacuated desiccator in order to remove the acetic acid as completely as possible. It is then suspended in an excess of acetone and placed in the refrigerator overnight. The filtration of the suspension yields a preparation, Fraction A, which contains 70 to 75 per cent of its P in the form of saponifiable P. The mother liquor is concentrated to dryness and dried again over NaOH in order to remove the last traces of acetic acid. This material is suspended in acetone and placed in the refrigerator overnight. Upon filtering, it yields Fraction B, which contains 75 to 80 per cent of its P in the form of saponifiable P.

Fractions A and B are separately treated in the following manner: Each substance is dissolved in a mixture of acetic acid and acetone (1:40) (1 gm. of substance in 20 cc. of solution) with slight warming. It is allowed to stand overnight at room temperature. The precipitate, which contains 60 per cent of its P in the form of saponifiable P, is filtered off. The filtrate is placed in the refrigerator overnight. The resulting precipitate is filtered and contains 75 to 85 per cent of its P in the form of saponifiable P. The mother liquor is brought to dryness by vacuum distillation and dried over NaOH. The resulting resinous material is suspended in acetone and placed in the refrigerator overnight. This fraction, which is small, contains 80 to 90 per cent of its P in the form of saponifiable P. This entire fractionation is repeated several times with fractions which contain over 80 per cent of their total P in the form of saponifiable P until a final substance is obtained containing over 95 per cent hydrolecithin.

The final dry substance is suspended in dry ether and kept in the refrigerator overnight. By this treatment, small amounts of a yellow pigment are removed from the substance. After filtration, the hydrolecithin is recrystallized either from diisobutyl ketone or from a mixture of acetone and glacial acetic acid (40:1). Due to the repeated fractionations our yields were low, about 2 gm. from 50 pounds of fresh lung.

Physical Properties of Hydrolecithin—Hydrolecithin is a white powder which crystallizes in needles from diisobutyl ketone. It crystallizes in aggregates of small needles from a mixture of 40 volumes of acetone and 1 volume of acetic acid. It is very soluble in alcohol, glacial acetic acid, very slightly soluble in acetone, insoluble in ether. The substance sinters to a clear viscous mass between 75–80° and melts at 237–238°. Two different samples of hydrolecithin gave iodine numbers of 3 and 4.6 respectively.

$C_{40}H_{82}O_9PN$. Calculated. C 63.9, H 10.9, N 1.86, P 4.1
Found. " 63.1, " 10.9, " 1.82, " 4.0

Specific rotation, +6.25° (4% solution of hydrolecithin in a mixture of chloroform-methanol, 1:1)

The substance forms with Reinecke acid a compound which is almost insoluble in alcohol, slightly soluble in acetone. It contains 4.68 per cent N and 3.12 per cent P. Hence, it is not a simple stoichiometric compound.

Products of Alkaline Hydrolysis of Hydrolecithin. Barium Salts of Fatty Acids—2 gm. of the powdered substance were refluxed for 4 hours with saturated aqueous $Ba(OH)_2$. After cooling, the barium soaps were filtered off and shaken with water in order to remove soluble impurities. After filtering and drying, the crude barium salt of the fatty acids weighed 1.7 gm.

Identification of Fatty Acid by Vacuum Distillation of Methyl Ester—1 gm. of barium soaps was refluxed in a 5 per cent methanolic solution of H_2SO_4 for 4 hours on a water bath. After removal of the $BaSO_4$ by filtration, the methyl esters were extracted from the acid methanolic solution with petroleum ether and distilled at a pressure of 0.007 mm. The methyl ester distilled at a fairly constant temperature. This is indicative of the presence of only one methyl ester. The distilled methyl ester was converted to the free acid and recrystallized several times to a constant melting point from petroleum ether at −20°; m.p. 62° (palmitic acid, 63°). The free acid had an iodine number of 0.5 and a molecular weight of 254 according to the results of its titration with N alkali (palmitic acid, 256).

Choline—The filtrate from the barium soaps was analyzed for choline,

total P, and glycerophosphate. Choline was determined by acidifying a cc. aliquot of the solution and adding ammonium reineckate. The characteristic crystals of choline reineckate formed rapidly in the solution. It was placed in the refrigerator overnight and filtered. 308 mg. of choline were found (theoretical, 323 mg.). Another small aliquot was analyzed for total P. Found, 77 mg. of P (theoretical, 82 mg.).

Barium Glycerophosphate—The largest part of the filtrate was used for the isolation of glycerophosphate. After its neutralization, basic lead acetate was added in slight excess. The precipitate was filtered over a Büchner funnel and carefully washed with water. It was decomposed by means of hydrogen sulfide. The filtrate from the lead sulfide was concentrated to a small volume. A hot saturated solution of barium hydroxide was added until approximately pH 9 was reached. The barium salt was precipitated by the addition of 3 volumes of alcohol. The barium salt was analyzed for glycerol according to the method of Blix and for P according to the method of Fiske and Subbarow.

Theoretical, glycerol, 30, P 10.1; found, glycerol, 28, P 9.4

DISCUSSION

Hydrolecithin (dipalmityl lecithin) as a constituent of animal lipides has not been known up to now. Its quantity in lung equals 20 to 40 per cent of the amount of the sphingomyelin present in this organ, since the crude ether-insoluble phosphatide mixture of sphingomyelin and hydrolecithin contains 20 to 40 per cent saponifiable, saturated monoaminophosphatide. The small final yield of pure dipalmityl lecithin is due to the substantial losses during the procedure applied for its isolation. In the process of fractionation of the ether-insoluble phosphatide mixture, no other saturated monoaminophosphatide was encountered but dipalmityl lecithin. This substance, according to its physical properties and the products of its hydrolysis, seems to be identical to the hydrolecithin isolated by Lesuk and Anderson (9) from *Cysticercus* larvae. In contrast to animal organs, hydrolecithin occurs in *Cysticercus* larvae as the only ether-insoluble phosphatide, since sphingomyelin was not found to be present in the ether-insoluble fraction. The physiological significance of hydrolecithins is not known. The occurrence in tissues of other saturated lipides such as dihydrocholesterol (11) and dihydrocerebrosides (dihydrosphingosine) (9, 12) indicates a process of the intermediary metabolism which leads to the formation of saturated lipides.

The natural hydrolecithin (dipamityl lecithin) found in lung is, in regard to melting point and fatty acids, not identical with hydrolecithin prepared by hydrogenation with colloidal palladium from unsaturated

lecithins of egg yolk (13–15) and of brain (16). We had the opportunity of comparing our hydrolecithin with an original sample of the synthetic distearyl lecithin of Grün and Limpächer (17, 18). The melting point of the latter was considerably lower (187°), while natural dipalmityl lecithin melts at 238°. Both substances sinter at 75–85° to a clear viscous mass.

SUMMARY

1. A method of isolation and crystallization of hydrolecithin from the ether-insoluble phosphatide mixture of lung is described.

2. The isolated saturated lecithin was identified by its split-products after alkaline hydrolysis as dipalmityl lecithin.

3. The amount of hydrolecithin present in the lung is 20 to 40 per cent of the sphingomyelin content of this organ.

BIBLIOGRAPHY

1. Levene, P. A., *J. Biol. Chem.*, **24**, 69 (1916).
2. Rosenheim, O., and Tebb, M. C., *J. Physiol.*, **41**, p. i (1910–11).
3. Walz, E., *Z. physiol. Chem.*, **166**, 210 (1927).
4. Thannhauser, S. J., and Setz, P., *J. Biol. Chem.*, **116**, 527 (1936).
5. Thannhauser, S. J., and Reichel, M., *J. Biol. Chem.*, **135**, 1 (1940).
6. Blix, G., *Mikrochim. acta*, **1**, 75 (1937).
7. Thannhauser, S. J., and Schmidt, G., in Annual review of biochemistry, Stanford University, **12**, 238 (1943).
8. Klenk, E., and Rennkamp, F., *Z. physiol. Chem.*, **267**, 145 (1941).
9. Lesuk, A., and Anderson. R. J., *J. Biol. Chem.*, **139**, 457 (1941).
10. Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 505 (1946).
11. Schönheimer, R., *Z. klin. Med.*, **123**, 749 (1933).
12. Carter, H. E., and Norris, W. P., *J. Biol. Chem.*, **145**, 709 (1942).
13. Paul, C., and Oehrne, H., *Ber. chem. Ges.*, **46**, 2 (1913).
14. Ritter, F., *Ber. chem. Ges.*, **47**, 530 (1914).
15. Levene, P. A., and West, C. J., *J. Biol. Chem.*, **33**, 111 (1918).
16. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **46**, 353 (1921).
17. Grün, A., and Limpächer, R., *Ber. chem. Ges.*, **58**, 1350 (1926).
18. Grün, A., and Limpächer, R., *Ber. chem. Ges.*, **60**, 147 (1927).

THE PREPARATION OF PURE SPHINGOMYELIN FROM BEEF LUNG AND THE IDENTIFICATION OF ITS COMPONENT FATTY ACIDS*

By S. J. THANNHAUSER, JOSEPH BENOTTI, AND NICHOLAS F. BONCODDO

(From the Boston Dispensary, the Joseph H. Pratt Diagnostic Hospital, and Tufts College Medical School, Boston)

It was demonstrated in the previous paper (1) that lung sphingomyelin, prepared by the usual methods of extraction with various solvents, contains hydrolecithin (dipalmityl lecithin) in appreciable quantity. Hydrolecithin, if present, invariably accompanies the sphingomyelin fraction. It has escaped discovery until recently because the physical properties of both substances, especially their insolubility in ether, are almost identical. For this reason it is necessary to modify the current procedures for the preparation of sphingomyelin (2–5) in order to obtain a sphingomyelin which is free of the saturated monoaminophosphatide. This was accomplished by a procedure in which crude sphingomyelin was treated with 0.25 N NaOH at 37° for 4 days, similar to the method described by Schmidt, Benotti, Hershman, and Thannhauser for the estimation of monoaminophosphatides and sphingomyelin in lipide mixtures (6). This pure sphingomyelin was used in our paper for the reinvestigation of the nature of the fatty acids combined with the amino group of its sphingosine component in acid amide linkage.

Sphingomyelin prepared with the older methods from brain, spleen, and liver yielded by acid hydrolysis palmitic, stearic, and lignoceric acids (2–5). Since it was found that hydrolecithin (dipalmityl lecithin) adheres to the sphingomyelin, the formation of palmitic acid during its acid hydrolysis may have originated from its hydrolecithin content and may not be a constituent of the sphingomyelin molecule itself in acid amide linkage. In the experiments described in this study it is shown, however, that lung sphingomyelin, even after it had been freed from dipalmityl lecithin, yielded palmitic and lignoceric acids in about equal quantities. Stearic acid was not found as a component of lung sphingomyelin.

It is possible that the fatty acid component of the sphingomyelin of other organs differs from the fatty acids of lung sphingomyelin. An investigation of the component fatty acids of hydrolecithin-free sphingo-

myelins prepared from brain, spleen, and liver is in progress in this laboratory.

Preparation of Sphingomyelin from Lung by Alkaline Saponification—The ether-insoluble lipide fraction was obtained from beef lung as described in the previous paper (1). The crude sphingomyelin was precipitated from the glacial acetic acid extract by acetone and dried after washing with acetone. According to the previous investigation this fraction, though completely free of unsaturated monophosphatides, consists of a mixture of sphingomyelin and hydrolecithin (dipalmityl lecithin). The proportion of these two components of this crude substance was 2:1 as shown by the results of the quantitative partition of the phospholipides (1, 6).

10 gm. of this material were suspended in a small amount of water and ground to a paste to which 200 cc. of 0.25 N NaOH were added. This suspension was shaken at 37° for 4 or 5 days. It was then acidified with glacial acetic acid and placed in the refrigerator overnight and filtered over Hyflo filter aid. After washing with acetone and finally with ether, the precipitate was extracted in a Soxhlet apparatus for 2 to 3 days with ether for the purpose of removing the fatty acids. The contents of the thimble which contained, besides sphingomyelin, small amounts of cerebrosides were dialyzed for 24 hours against running water for the purpose of removing inorganic material. The dialyzed suspension was filtered over Hyflo filter aid and washed with acetone. The sphingomyelin was extracted from the dried residue by means of petroleum ether-methanol (9:1). The extract was filtered, concentrated to a small amount of thin syrupy consistency, and precipitated with a large excess of acetone (1000 to 1500 cc.). The precipitate contains almost the total amount of sphingomyelin (3 to 4 gm.). Since this substance still gives a slight Molisch test, it is taken up in petroleum ether-methanol (9:1) and put through a chromatographic column of Al_2O_3 for the selective adsorption of cerebrosides (5). The sphingomyelin is recovered from the concentrated solution by precipitation with acetone and recrystallized from hot ethyl acetate. The analysis of the substance gave the following results: N, 3.4, P 3.58 per cent; N:P ratio 2:1.[1]

Properties of Sphingomyelin from Lung—A white crystalline substance was obtained which is easily soluble in benzene, soluble in warm alcohol and hot ethyl acetate, but insoluble in ether and acetone. It can be recrystallized from hot ethyl acetate. M.p. 209°; $[\alpha]_D^{29} = +6.25°$ (4 per

---

[1] The amount of saponifiable P (1) of the material is usually less than 1 per cent of the total P. If it is higher, the saponification in 0.25 N NaOH at 37° is repeated.

cent solution of sphingomyelin in a mixture of chloroform-methanol 1:1). Iodine number, 30 (theoretical, 30.6). This iodine number indicates the presence of one double bond in the sphingomyelin molecule. Since sphingosine contains one double bond, the presence of unsaturated fatty acids is thus excluded.

The substance is precipitated from its methanolic solution by Reinecke acid. The crystalline reineckate contains 5.22 per cent N and 3.09 per cent P, and hence is not a simple stoichiometric compound. The reineckate is insoluble in alcohol and acetone.

Preparation of Sphingomyelin by Exhaustive Extraction with 97 Per Cent Acetone—Sphingomyelin can also be separated almost completely from hydrolecithin by exhaustive extraction of the crude fraction with 97 per cent acetone (97 volumes of acetone and 3 volumes of water). The crude material (see above) is mixed with sand and extracted with 97 per cent acetone in a Soxhlet apparatus for 3 weeks. Following this extraction, the residue in the thimble is taken up in 1000 cc. of a mixture of petroleum ether and methanol (9:1) and filtered. The filtrate is concentrated under reduced pressure to a volume of 5 to 10 cc. and completely precipitated by adding an excess of acetone. The sphingomyelin is recrystallized from hot ethyl acetate. Analysis, 3.25 per cent N, 3.8 per cent P (N:P = 2:1). The saponifiable P of this substance is 5 per cent of the total P. The yield of sphingomyelin obtained according to this procedure is small, since a considerable part of the sphingomyelin is extracted by the acetone together with the hydrolecithin. On cooling the acetone extract, a mixture of hydrolecithin and sphingomyelin crystallizes in long needles. Attempts to recover pure sphingomyelin from this mixture by extraction with 97 per cent acetone were not successful.

Because of the small yields, the isolation of sphingomyelin by means of continuous extraction with 97 per cent acetone cannot be recommended for the preparation of large amounts of this lipide. The method, however, is of principal importance, as it demonstrates the possibility of preparing sphingomyelin almost free of hydrolecithin without the application of mild saponification.

Fatty Acids of Pure Sphingomyelin Prepared from Lung—9 gm. of sphingomyelin obtained by the saponification procedure were refluxed with 300 cc. of a solution of 10 per cent sulfuric acid in methanol. After cooling, the methyl esters were extracted with successive portions of petroleum ether. After concentrating the combined petroleum ether fractions to dryness, 2.65 gm. of methyl esters were obtained. The methyl esters were fractionated by vacuum distillation (2) at a pressure of 0.009 mm. Four fractions were obtained: Fraction 1, liquid, 0.58 gm.,

and Fraction 2, viscous liquid, 0.45 gm., distilled between 92–102° at 0.009 mm.; Fraction 3, solid, 0.25 gm., distilled up to 121°; Fraction 4, solid, 1.27 gm., distilled between 120–135°.

Fractions 1 and 2 were combined and redistilled. The temperature rose to a level of 96° until the distillation was completed. This behavior during the distillation indicated the presence of only one methyl ester. It was converted to the free acid by saponification with N aqueous sodium hydroxide. The acid was recrystallized from petroleum ether at −10°. M.p. 62°; molecular weight by titration, 260; iodine number, 1. It is evident that the acid is identical with palmitic acid (m.p. 62°, molecular weight, 256).

Fraction 4 was not redistilled but converted directly to the free acids. The substance was recrystallized several times from petroleum ether at room temperature. M.p. 82°; molecular weight by titration, 373; iodine number, 1.7. The acid is thus identical with lignoceric acid (m.p. 84°; molecular weight, 368).

Fraction 3 was converted to the free acid and pooled with all the mother liquors from the petroleum ether recrystallizations of Fraction 4. These fractions were recrystallized from petroleum ether at room temperature for the purpose of removing the greater part of lignoceric acid. The soluble fraction was remethylated and distilled. The two fractions thus obtained were palmitic acid and lignoceric acid. M.p. of lignoceric acid fraction 81°; m.p. of palmitic acid fraction 60°.

If stearic acid had been present in measurable amounts, it would have been found, as small quantities of known mixtures of palmitic and stearic acids have been separated by the method employed (7). For this reason it may be concluded that stearic acid is not a constituent of sphingomyelin prepared from beef lung.

The purification of the free acids was carried out by means of recrystallization from petroleum ether at different temperatures. Lignoceric acid crystallizes readily at 0°, while palmitic acid comes out only at much lower temperatures (−10° to −20°).

Acid hydrolysis with 10 per cent H_2SO_4 in methanol was also carried out on 4.5 gm. of a sphingomyelin sample which was freed from hydrolecithin by exhaustive extraction with 97 per cent acetone, but not by mild saponification. The acid hydrolysis of this preparation also yielded lignoceric acid and palmitic acid in approximately equal amounts.

SUMMARY

1. It was found that sphingomyelin prepared according to all current methods was a mixture in various proportions of sphingomyelin and hydrolecithin. This is explained by the strong resemblance of the physical properties and especially by the insolubility in ether of both substances.

2. Two methods for the preparation of lung sphingomyelin free of hydrolecithin have been described.

3. Sphingomyelin prepared from beef lung is a mixture of palmityl and lignoceryl sphingomyelin. Stearic acid was found to be absent in lung sphingomyelin.

BIBLIOGRAPHY

1. Thannhauser, S. J., Benotti, J., and Boncoddo, N. F., *J. Biol. Chem.*, **166,** 669 (1946).
2. Levene, P. A., *J. Biol. Chem.*, **24,** 69 (1916).
3. Rosenheim, O., and Tebb, M. C., *J. Physiol.*, **41,** p. i (1910–11).
4. Walz, E., *Z. physiol. Chem.*, **166,** 210 (1927).
5. Thannhauser, S. J., and Setz, P., *J. Biol. Chem.*, **116,** 527 (1936).
6. Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **166,** 505 (1946).
7. Fränkel, E., Bielschowsky, F., and Thannhauser, S. J., *Z. physiol. Chem.*, **218,** 1 (1933).

THE LIPIDES OF BLOOD, LIVER, AND EGG YOLK
OF THE TURTLE

By I. L. CHAIKOFF and C. ENTENMAN

(From the Division of Physiology, University of California Medical School, Berkeley)

Although the concentration of lipides in the blood and liver of the male and immature female bird does not differ greatly from that found in other species, such as man and dog, avian lipide metabolism differs from mammalian in being under the control of ovarian hormones (1, 2). This distinctive feature of the lipide metabolism of the bird has been established by the work of several laboratories (3–5). Thus in the bird actively engaged in egg laying there occurs a rise in the levels of fat, phospholipides, and cholesterol of the blood; in this state total lipides of the blood may rise as high as 4700 mg. per 100 cc. as compared with about 500 mg. in the non-laying state. It has since been shown that this rise in the various lipide constituents of the blood could be reproduced in the immature female bird by stimulation of ovarian activity by the injection of pregnant mare serum (6). Finally it has been demonstrated that such crystalline estrogenic compounds, as estrone, estradiol, and stilbestrol increase the blood lipides of the male bird as well as of the immature female (2). In all experiments in which pregnant mare serum and crystalline sex hormones were injected into immature female birds, the rise in the blood lipides was found to be closely correlated with growth of the oviduct, a tissue that responds in this way to increases in estrogenic activity.

The present investigation was suggested by the similarity in structure and development between bird and reptile. Indeed, the bird has been described as a "glorified reptile" (7). Birds and most reptiles are unique in being provided with cleidoic eggs. Such eggs are walled off from their environment by a firm shell or membrane and contain yellow yolks which, enclosed in sacs connected to the digestive tract, serve to supply the entire needs of the growing embryo except oxygen (8).

EXPERIMENTAL

Upon arrival in Berkeley the turtles were placed in a pond where they were kept for several weeks before being sacrificed. During their stay here they were supplied with no food other than the débris that accumulates in pond water.

The methods employed for the determination of the various lipide constitutents have been described elsewhere (9).

Results

The blood lipides of 103 turtles were measured. The turtles were classified in four groups: males, females containing inactive ovaries, females containing active ovaries that showed no evidence of ovulation, females containing active ovaries as well as eggs in their oviducts. For the purpose of this study an active ovary is defined as one that contained one or more ova larger than 5 mm. in diameter.

The mean values and their standard errors for cholesterol (total, free and ester), total fatty acids, phospholipides, and total lipides of whole blood are presented in Table I. Values for plasma are recorded in Table II.

In order to determine whether the differences in blood lipide levels observed in relation to ovarian activity were significant, the differences among the means were tested by Fisher's *t* ratios (10). The results are recorded in Table III.[1] No significant differences for any of the lipide constituents were found between male turtles (Group I) and female turtles containing inactive ovaries (Group II). Highly significant differences (at or below the 1 per cent level of certainty) were demonstrated between males on the one hand (Group I) and females with active ovaries (Groups III and IV) on the other hand. Furthermore, highly significant differences were observed for all lipide constituents between Group II (females with inactive ovaries) and Group IV (females that contained active ovaries and eggs in their oviducts).

Although significant differences for total fatty acids and phospholipides were found between Groups II and III, acceptable significant differences were not demonstrated between these two groups for any of the cholesterol constituents. As noted above, the arbitrary criterion chosen to separate Groups II and III was the size of the ova, namely 5 mm. If the arbitrary criterion had been set at a lower value, it is possible that significant differences would have been obtained for all lipide constituents.

No significant differences were demonstrated (a) between Groups I and II and (b) between Groups III and IV. A summation was therefore made of Groups I and II and this new group designated Group V; similarly Group VI represents the summation of Groups III and IV. The *t* values for the differences between Groups V and VI leave no doubt of an effect of ovarian activity upon blood lipides.

Liver Lipides—The livers of forty-two turtles were analyzed for total fatty acids, phospholipides, and free and esterified cholesterol. The mean values and their standard errors are recorded in Table IV. No striking differences were demonstrated among the three groups, males, females with inactive ovaries, and females with active ovaries.

[1] We are indebted to Mr. R. Skahen for the statistical treatment of the data presented here.

TABLE I

Whole Blood Lipides of Turtles (Chrysemys picta bellii)

Group No.	Classification	No. of turtles	Turtle size		Ovarian activity*			Cholesterol			Total fatty acids	Phospho-lipides	Total lipides
			Length	Weight	Ovary		No. of eggs in oviduct	Total	Free	Ester			
					Diameter of largest ovum	No. of ova in each turtle larger than 5 mm. in diameter							
			cm.	gm.	mm.			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
I	Males	32	13.4 ± 1.1†	374 ± 78				90 ± 31	49 ± 13	39 ± 23	232 ± 69	195 ± 43	322 ± 93
II	Females with inactive ovaries‡	20	12.6 ± 0.7	327 ± 74	5	None	None	110 ± 37	52 ± 12	53 ± 25	259 ± 46	195 ± 47	369 ± 71
III	Females with active ovaries‡ but no evidence of ovulation	33	16.8 ± 2.0	654 ± 33	20§ 10‖	40§ 10‖	"	127 ± 34	66 ± 20	61 ± 24	340 ± 107	240 ± 59	468 ± 121
IV	Females with active ovaries and eggs in oviduct	18	18.5 ± 1.6	727 ± 132	¶	¶	18§ 2‖	150 ± 29	67 ± 14	86 ± 20	327 ± 59	251 ± 30	487 ± 86
V	Males and females with inactive ovaries	52						100 ± 33	51 ± 13	46 ± 23	245 ± 62	195 ± 45	345 ± 86
VI	Females with active ovaries with and without evidence of ovulation	51						138 ± 33	66 ± 18	74 ± 23	333 ± 94	246 ± 52	477 ± 112

* These studies were carried out during the months of May and June.   † Standard error.
‡ Inactive ovaries have been arbitrarily defined as those containing no ova larger than 5 mm. in diameter; an active ovary as one containing one or more ova having a diameter larger than 5 mm.   ¶ The ovaries of these turtles contained many ova that had a diameter of 15 mm.
§ Maximum.   ‖ Minimum.

TABLE II

Total Fatty Acid Content of Turtle Plasma (Pseudemys scripta)

No. examined	Length	Hematocrit	Total fatty acids
	cm.	*per cent*	*mg. per 100 cc.*
15*	18.7 ± 2.0†	17.5 ± 5.3	196 ± 66

* Six males plus nine females with inactive ovaries.
† Standard error.

TABLE III

t Values of Differences among Whole Blood Lipide Levels of Experimental Groups
   With the number of cases* used here, a *t* value of 3.69 is significant at the 0.1 per cent level of certainty, 2.77 at the 1 per cent level, and 2.47 at the 5 per cent level.

Group No.	Description	Lipide constituent	Compared with			
			Group I	Group II	Group III	Group V
I	Males	Cholesterol, total				
		" free				
		" ester				
		Total fatty acids				
		Phospholipides				
II	Females with inactive ovaries	Cholesterol, total	2.05			
		" free	0.72			
		" ester	1.89			
		Total fatty acids	1.63			
		Phospholipides	0.00			
III	Females with active ovaries but no evidence of ovulation	Cholesterol, total	2.79	1.61		
		" free	3.76	2.61		
		" ester	3.63	1.15		
		Total fatty acids	4.81	3.13		
		Phospholipides	3.45	2.82		
IV	Females with active ovaries and eggs in oviduct	Cholesterol, total	6.28	3.44	2.30	
		" free	4.05	3.12	0.19	
		" ester	6.86	4.25	3.64	
		Total fatty acids	4.81	3.85	0.47	
		Phospholipides	4.59	3.99	0.73	
V	Males and females with inactive ovaries	Cholesterol, total				
		" free				
		" ester				
		Total fatty acids				
		Phospholipides				
VI	Females with active ovaries with and without evidence of ovulation	Cholesterol, total				5.73
		" free				4.79
		" ester				5.87
		Total fatty acids				5.59
		Phospholipides				5.22

* For the number of cases in each group, see Table I.

Yolk Lipides—The cholesterol, phospholipide, and total fatty acid contents of the yolks of turtles are recorded in Table V and of the domestic fowl

TABLE IV

Liver Lipide of Turtle (Chrysemys picta bellii)

All lipide values expressed as per cent of wet weight of tissue.

Classification	No. examined	Turtle size		Liver weight	Cholesterol			Total fatty acids	Phospholipides
		Length	Weight		Total	Free	Ester		
		cm.	*gm.*	*gm.*					
Males	15	12.8	316	19.5	0.35	0.18	0.19	4.4	1.0
		±0.8*	±49	±3.4	±0.40	±0.03	±0.14	±3.7	±0.30
Females with inactive ovaries	14	12.8	333	16.2	0.53	0.19	0.34	5.1	1.3
		±0.8	±108	±4.7	±0.27	±0.03	±0.27	±3.0	±0.10
Females with active ovaries and eggs in oviduct	13	15.7	625	32.4	0.48	0.19	0.29	6.0	1.4
		±1.6	±106	±10.3	±0.13	±0.02	±0.14	±3.1	±0.10

* Standard error.

TABLE V

Lipide Content of Yolks Removed from Ovaries of Turtles (Chrysemys picta bellii)

All lipide values expressed as per cent of wet weight of tissue.

No. examined	Size		Diameter of largest yolk	Cholesterol			Total fatty acids	Phospholipides	Total lipides
	Length	Weight		Total	Free	Ester			
	cm.	*gm.*	*mm.*						
13	16.2	602	15	0.57	0.41	0.16	14.2	2.0	14.8
	±2.3	±136		±0.10*	±0.01	±0.10	±2.6	±0.4	±2.6

* Standard error.

TABLE VI

Lipide Content of Chicken Yolks

Values expressed as per cent of wet weight.

No. examined	Cholesterol			Total fatty acids	Phospholipides	Total lipides
	Total	Free	Ester			
5	2.01 ± 0.04	1.87 ± 0.17	0.16 ± 0.29	27.90 ± 0.67	7.56 ± 0.54	29.74 ± 0.71

(white Leghorns) in Table VI. It is of interest that the fatty acid content of the turtle yolk is lower than that of the bird yolk. Phospholipides represented approximately 30 per cent of the total lipides in the bird yolk but only 15 per cent in the turtle yolk.

Table VII shows that the entire turtle ovary may accumulate as much as 6 gm. of fatty acids and 1.6 gm. of cholesterol.

DISCUSSION

The results of the present investigation demonstrate that ovarian activity in the female turtle, as judged by the presence in the ovary of numerous large ova, is accompanied by a rise in the content of lipides in the blood. The most pronounced rise occurred in neutral fat, although statistically significant changes were also observed in phospholipides and cholesterol; in this respect the turtle resembles the bird. The response of lipide metabolism to ovarian activity in the turtle differs, however, from that in the bird

TABLE VII

Lipide Content of Whole Ovary of Turtles

All lipide values expressed as per cent of wet weight of tissue.

Turtle No.	Type	Turtle length	Total weight of ovary	Total cholesterol	Total fatty acids	Phospho-lipides	Total lipides
		cm.	gm.				
73*	*Chrysemys picta bellii*	5	4.1	0.44	10.6	2.3	11.7
201†	*Pseudemys scripta*	8.5	36.4	0.45	17.0		17.5
203‡	" "	6.5	23.7	0.48	12.6		13.1

* The ovary of this turtle contained six ova measuring 7.5 mm. in diameter and many ova of lesser diameters.

† The ovary of this turtle contained seven ova measuring 22 mm. in diameter and many ova of lesser size.

‡ The ovary of this turtle contained eight ova measuring 15 mm. in diameter, nine ova with diameters of 10 mm., and many ova that had diameters less than 10 mm.

(1) in two respects: (*a*) the blood lipides of the laying bird are higher and (*b*) there is no pronounced increase in the fat content of the liver of the laying turtle.

That lipide metabolism in the bird and turtle possesses a common distinguishing characteristic not found in the mammal is of particular interest, for, as noted above, birds and reptiles are very closely related in their ovulation and phylogeny. Inasmuch as the ova of birds and turtles are rich in fat (those of the turtle may contain as much as 19 per cent fatty acids), it is reasonable to believe that the rise in blood lipides during ovarian activity represents a mechanism that makes possible a rapid mobilization of fat for use in yolk formation.

SUMMARY

1. Values for fatty acids, cholesterol, and phospholipides of blood, liver, and yolk of the turtle are presented.

2. The levels of these three lipide constituents in the blood of the turtle, as in the case of the bird, are related to ovarian activity.

BIBLIOGRAPHY

1. Lorenz, F. W., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **122,** 619 (1937–38). Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **123,** 577 (1938).
2. Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, **134,** 495 (1940).
3. Landauer, W., Pfeiffer, C. A., Gardner, W. U., and Man, E. B., *Proc. Soc. Exp. Biol. and Med.*, **41,** 80 (1939).
4. Zondek, B., and Marx, L., *Arch. internat. pharmacod.*, **61,** 77 (1939); *Nature*, **143,** 378 (1939).
5. Riddle, O., *Endocrinology*, **31,** 498 (1942).
6. Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, **126,** 133 (1937–38).
7. Romer, A. S., Man and the vertebrates, Chicago, 99 (1945).
8. Needham, J., Biochemistry and morphogenesis, London, 48 (1942).
9. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **106,** 267 (1934).
10. Fisher, R. A., Statistical methods for research workers, Edinburgh, 9th edition, 114 (1944).

FATTY ACID METABOLISM

V. THE CONVERSION OF FATTY ACID INTERMEDIATES TO CITRATE, STUDIED WITH THE AID OF ISOTOPIC CARBON*

By SIDNEY WEINHOUSE, GRACE MEDES, AND NORMAN F. FLOYD

(From the Research Laboratories of the Houdry Process Corporation of Pennsylvania, Marcus Hook, and the Lankenau Hospital Research Institute, Philadelphia)

The formation of citric acid by various animal tissues in the presence of acetoacetate and oxalacetate (2–4), and the incorporation of isotopic carbon in α-ketoglutarate, succinate, and fumarate during the oxidation of C^{13}-labeled acetate and acetoacetate by rat and guinea pig kidney (5, 1) suggests that the tricarboxylic acid cycle provides a pathway for the oxidation of fatty acids as well as carbohydrate. This concept has been disputed, however, by Weil-Malherbe (6) and Krebs (7).

To obtain direct information concerning the participation of tricarboxylic acids in the oxidation of fatty acid intermediates, a study was made of citrate formation during the biological oxidation of acetate (8) and acetoacetate, labeled with C^{13}.

EXPERIMENTAL

Carboxyl-Labeled Sodium Acetate—Isotopic CO_2 was liberated from 50 mM of barium carbonate and collected in a high vacuum system where it was purified and dried by low temperature sublimation. It was then released into an evacuated flask containing 80 mM of methylmagnesium iodide, and the flask shaken until absorption was complete. The reaction mixture was decomposed with dilute H_2SO_4, steam-distilled, and the distillate neutralized, evaporated, reacidified, and redistilled with steam in the presence of a little silver sulfate to remove HI. The distillate was neutralized with standard, CO_2-free NaOH and evaporated to dryness *in vacuo*. The yield of sodium acetate ranged, in several such preparations, between 92 and 96 per cent.

Carbonyl- and Carboxyl-Labeled Sodium Acetoacetate—A mixture of 46 mM of isotopic sodium acetate and 50 mM of diethyl sulfate was heated at 145° for 2 hours in a sealed tube. The product was fractionated under reduced pressure, yielding 42.3 mM of ethyl acetate; n_D^{20} 1.3722; recorded, 1.3722.

The ethyl acetate was distilled into a glass tube containing 21 mM of dry sodium ethoxide; the tube was then sealed and heated for 16 hours at 108°. The unchanged ethyl acetate (14.5 mM) was distilled off *in vacuo;* the residue was then decomposed with 2 N H_2SO_4 and extracted with ether. The dried ether extract was evaporated and the residual oil fractionated under reduced pressure, yielding 863 mg. of a colorless liquid, n_D^{20} 1.4127; recorded for ethyl acetoacetate, 1.4209. Assay of this material for volatile acid and acetone by the Van Slyke (9) procedure indicated it was a mixture of 83 mole per cent ethyl acetoacetate and 17 mole per cent ethyl acetate. Owing to the small quantity available, it was used without further purification. The acetone, isolated as the Denigès complex, had a C^{13} excess of 2.16 per cent, corresponding to an excess of 6.48 atom per cent in the carbonyl and carboxyl carbons.

Immediately before using, the ester was saponified by dissolving in the calculated quantity of 0.1 N NaOH, allowed to stand at room temperature for 24 hours, and the solution evaporated to dryness at room temperature to remove alcohol.

Other Substrates—Oxalacetic acid was prepared by the method of Schneider (10); m.p. 148–150°. *cis*-Aconitic anhydride, m.p. 75°, was prepared by the method of Malachowski and Maslowski (11). Other substrates were commercial products.

Incubation of Carboxyl-Labeled Acetate with Non-Isotopic Citrate— Tissue slices and minces were prepared as described previously (12). The substrates were made up to 100 ml. with Krebs' Ringer-phosphate (13) and 10 ml. removed for acetate (14) and citrate (15) determinations. The remaining 90 ml. were distributed equally among three 125 ml. Warburg type flasks, each containing approximately one-third of the tissue preparation. The central well held a small glass tube containing a CO_2-free alkali-soaked filter roll. All three flasks were connected to a single gas burette; oxygen consumption was measured volumetrically at atmospheric pressure. The flasks were filled with oxygen and, after equilibration, shaken for 2 hours at 37.5°. Acid (0.75 ml. of 2 N H_2SO_4) was then tipped in from the side bulb and shaking continued an additional 15 minutes. The tubes containing the filter rolls were quickly transferred, under nitrogen, to another flask, and the CO_2 liberated by acid, measured, and swept, with nitrogen, into an absorption tube containing CO_2-free alkali from which the CO_2 was ultimately liberated for determination of its C^{13} content.

The solutions from the three flasks were combined, and the tissues washed successively with dilute alkali and water; the solution and washings were brought to a definite volume, and, following removal of an aliquot for citrate determination, they were steam-distilled with 50 gm. of magnesium sulfate. The volatile acid thus recovered was redistilled, neutralized

with CO_2-free NaOH, and the sodium acetate recovered by evaporation to dryness.

The residue from the first distillation was extracted continuously with ether for at least 72 hours, the ether extract was evaporated, and the residue dissolved in water. About 70 to 90 per cent of the citric acid was extracted by this procedure. The silver salts were precipitated (16), decomposed with H_2S, and the clear filtrate neutralized and evaporated to 0.5 ml., methanol added, and the crude trisodium citrate recrystallized from aqueous methanol. The oxidation and degradation procedures employed for determining the content and distribution of C^{13} were the same as those described below in connection with the experiments with isotopic acetoacetate.

Citric Acid Production by Kidney Homogenate—Whole rat kidneys were homogenized at $0°$ in an all-glass Potter-Elvehjem homogenizer with an approximately equal quantity of Krebs' phosphate-saline solution buffered at pH 7.4. Details for the individual experiments are recorded in Table II. Ketone bodies were determined by the Van Slyke method (9) and citrate by the method of Pucher (15).

Incubation of Oxalacetate and Isotopically Labeled Acetoacetate—Kidneys from seven rats, fasted 24 hours, were weighed and homogenized with 9 ml. of ice-cold Krebs' phosphate-saline, pH 7.4. The homogenized tissue was strained through cheese-cloth and distributed equally among three flasks, each containing approximately 0.15 mM of labeled acetoacetate and 0.5 mM of $BaCl_2$ in 7.5 ml. of saline. Then 0.3 mM of freshly neutralized sodium oxalacetate was added, and the flasks shaken for 2 hours at $37.5°$. Oxygen uptake was measured and the respiratory CO_2 collected as described above. The contents of the flasks were pooled, centrifuged, and the tissue washed successively with 0.03 M NaOH and water. The supernatant and washings were brought to a volume of 75 ml. and a 5 ml. aliquot removed for citrate and keto acid determinations. The remainder was acidified with 1 ml. of 18 N H_2SO_4 and steam-distilled. The distillate was neutralized and redistilled with steam; the neutral volatile fraction thus obtained was heated according to the Van Slyke procedure for recovery of the acetone formed by the thermal decarboxylation of the unutilized acetoacetate. The residue from this second distillation was acidified and redistilled to recover the small quantity of acetate present originally in the labeled acetoacetate.

Isolation of Citric Acid—The residue from the first distillation was combined with similar residues obtained from two subsequent experiments and extracted continuously with ether for 72 hours. The ether extract was evaporated, yielding a residue containing 9.5 mM of carbon, having a

C^{13} excess of 0.26 per cent, of which 84.3 mg. were citric acid.   The solution was made to 30 ml.; 10 ml. of 10 per cent silver nitrate were added, and NH_4OH added to a purple color with brom-cresol purple.   There were obtained 304 mg. of silver salts with a C^{13} excess of 0.39 per cent.   The silver salts were decomposed with H_2S, and the clear filtrate, containing 83.0 mg. of citric acid, was evaporated to 7 ml.   To the hot solution were added 250 mg. of quinidine, bringing the pH to 6.5.   After cooling to room temperature, the turbid solution was filtered without washing, and the filtrate seeded with a crystal of quinidine citrate.   After standing at room temperature for 48 hours, the crystals of quinidine citrate, characteristically separating in rosettes of fine, slender prisms, were filtered, washed with two 1 ml. portions of water, and dried in a high vacuum. There were obtained 155 mg. of colorless salt, which softened at 128–130° and melted completely at 134°.   The same behavior was found for "synthetic" quinidine citrate; C found, 63.2 per cent; found for "synthetic" quinidine citrate, 63.5; calculated for $3C_{20}H_{24}O_2N_2 \cdot 2C_6H_8O_7$, 63.7.

On evaporation of the filtrate to 4 ml. and addition of 50 mg. more of quinidine, an additional 45 mg. of quinidine citrate were obtained, melting at 125–130°.   Thus a total of 203 mg. of quinidine salt was isolated, representing 57.5 mg. of citric acid, or 68 per cent of the amount extracted.

100 mg. of the quinidine salt were dissolved in 15 ml. of hot water, ammonia was added in excess, and the free quinidine extracted with chloroform.   The aqueous layer was treated with $AgNO_3$, yielding 55.5 mg. of silver citrate.   Its C^{13} excess was 0.76 atom per cent.

Degradation of Citric Acid—40 mg. of quinidine citrate were placed in a glass tube carrying a small side bulb in which was placed 1 ml. of concentrated H_2SO_4.   The tube was attached to the vacuum line by means of an adapter consisting of a stop-cock with ground joints on both ends, one fitting the glass tube, the other for attachment to the vacuum system. This arrangement allowed removal of the tube from the vacuum line without breaking the vacuum.   After evacuating, the assembly was detached, cooled to −10°, and the sulfuric acid tipped in.   The tube was shaken at −10° until the quinidine citrate dissolved, then taken from the cold bath, and allowed to stand at room temperature until visible bubbling ceased (3 hours).   The tube was attached to the vacuum line and the evolved gas transferred by means of a Töpler pump to a sample tube for mass spectrometric determination.   The gas consisted of carbon monoxide 91.8, carbon dioxide 1.5, and air 5.7 per cent.

The tube was then reevacuated and heated for 2 hours at 100°.   The carbon dioxide evolved was condensed in the vacuum line and sublimed into a sample tube for C^{13} analysis.

The three non-carboxyl carbons of citric acid were isolated as ace-

tone by oxidation of a separate portion of citric acid by means of dilute potassium dichromate. In the course of a study of interfering substances in the Van Slyke determination of β-hydroxybutyrate, it was found that citric acid, under these conditions, gives a 60 to 70 per cent yield of acetone as the Denigès complex (identified by iodine titration and preparation of derivatives).

Results

Oxidation of Isotopic Acetate by Liver and Kidney in Presence of Citrate— In an attempt to demonstrate the intermediary formation of tricarboxylic acids from acetate, rat liver slices and kidney slices and mince were incubated in oxygen with a mixture of carboxyl C^{13}-labeled acetate and nonisotopic citrate, the latter in sufficient quantity to allow isolation at the close of the experiment. It would be expected, under these circumstances, that isotopic tricarboxylic acids, if formed, would come into equilibrium with added citrate, resulting in an enrichment of C^{13} in the recovered citrate.

As shown in Table I, however, the citrate isolated from such experiments had a normal C^{13} content within the ± 0.02 per cent error of the mass spectrometric measurements. In the last experiment, in which *cis*-aconitate was substituted for citrate, the action of aconitase resulted in its conversion to citrate, which likewise contained no appreciable excess C^{13}. Inasmuch as any istopic carbon would have been expected to be found in the carboxyl carbons, the sodium citrate was subjected to a degradation procedure involving reaction with concentrated sulfuric acid (17). In the cold, cleavage of the tertiary carboxyl occurs quantitatively to yield CO and acetonedicarboxylic acid; the latter on subsequent heating breaks down to carbon dioxide and condensation products of acetone. By analyzing the CO and CO_2 separately, it was possible to obtain C^{13} determinations on each of the two types of carboxyl carbons. In none of the four experiments in Table I did either the CO from the tertiary COOH or CO_2 from the primary carboxyls contain a significant excess of C^{13}.

In each experiment there was an active utilization of acetate, part of which was completely oxidized, as indicated by the presence of excess C^{13} in the respiratory CO_2. The respiratory CO_2 accounted for only 25, 56, 44, and 44 per cent respectively of the C^{13} utilized, however. The fate of the C^{13} unaccounted for is unknown as yet, though from the recent report of Bloch, Borek, and Rittenberg (18) part may be expected to have been incorporated in various tissue constituents.

It is noteworthy that apparently no acetate was produced in these experiments, the C^{13} content of the acetate recovered being virtually the same as at the start.

Though the results indicate that acetate oxidation can occur without formation of intermediates which are in equilibrium with added tricarboxylic acids, they do not necessarily prove that tricarboxylic acids are not formed, since only positive results in this type of experiment are conclusive.

TABLE I

C^{13} Distribution after Incubating Mixtures of Citrate and C^{13} Carboxyl-Labeled Acetate with Rat Liver and Kidney, 2 Hours in O_2 at 37.5° in 90 Ml. of Krebs' Saline-Phosphate Solution

	Liver slices, 3.75 gm.		Kidney slices, 3.03 gm.		Kidney mince, 5.25 gm.		Kidney mince + cis-aconitate, 4.50 gm.	
	m_M	C^{13} excess	m_M	C^{13} excess	m_M	C^{13} excess	m_M	C^{13} excess
Acetate, start................	0.789	3.90	0.815	3.90	0.806	3.90	0.764	3.90
" recovered...........	0.610	3.88	0.583	3.87	0.496	3.75	0.602	3.88
" utilized.............	0.179		0.232		0.310		0.162	
Citrate, start................	0.630	0.00	0.615	0.02	0.635	0.01	cis-Aconitate 0.450	0.00
" recovered...........	0.489	0.01	0.388	0.01	0.362	0.02	0.265	0.02
" utilized.............	0.141		0.227		0.373		0.185	
" primary carboxyls...		−0.01		0.01		0.04		0.00
" tertiary carboxyl*...		−0.03		0.01		0.02		0.02
O_2 consumed................	0.719		0.976		1.395		0.856	
CO_2 evolved................	0.516	0.69	1.018	1.00	1.232	0.86	0.777	0.71

* The CO is not entirely pure, being contaminated with small quantities, <10 per cent, of air and CO_2. Since nitrogen and its N^{15} isotope and CO ions formed by the breakdown of CO_2 contribute to the masses 28 and 29, it is necessary to make corrections for these substances in the calculation of the C^{13} ratio of the CO. The procedure followed was to scan the entire spectrum, from which the corrected 28 and 29 peaks were calculated by means of the following equations: (a) observed 28 peak $= X + 0.12y + 4.9z$; (b) observed 29 peak $= X' + 0.12y' + 0.041z$, where $X =$ the contribution of $C^{12}O$ to the 28 peak, $X' =$ the contribution of $C^{13}O$ to the 29 peak, $y =$ the observed 44 peak due to $C^{12}O_2$, $y' =$ the observed 45 peak due to $C^{13}O_2$, and $z =$ the observed 32 peak due to oxygen. The coefficients represent the relative contribution of air and CO_2 to the 28 and 29 peaks, as determined from the mass patterns of the pure gases.

Production of Citric Acid by Rat Kidney Homogenates—Inasmuch as these isotope dilution experiments were inconclusive concerning the participation of tricarboxylic acids in acetate metabolism, a study was made of the Breusch-Wieland system, directed primarily toward obtaining sufficient citric acid for isolation in pure form. In contrast with the results of Breusch with cat kidney (19) and Wieland and Rosenthal with cattle kidney (3), we found that rat kidney mince, despite its active utilization of acetate and acetoacetate, produced only small quantities of citrate when

these substances were incubated together with oxalacetate. This may be attributed to the high metabolic activity of citrate in these preparations (*cf.* Table I). With finer subdivision of the tissue, however, *i.e.* homogenization by the Potter-Elvehjem technique, high yields of citrate were obtained from oxalacetate and acetoacetate.

The production of citric acid by rat kidney homogenates in the presence of various substrates is shown in Table II. Though small quantities of citrate are formed when acetate, acetoacetate, and oxalacetate are present separately, highest yields were observed when acetoacetate and oxalacetate were present together. About one-third of the utilized acetoacetate was recovered as β-hydroxybutyrate. There is no apparent utilization of ace-

TABLE II

Citric Acid Formation by Rat Kidney Homogenate

Substrates in 0.01 M final concentration in 33 ml. of Krebs' phosphate-saline incubated 2 hours in O_2 at 37.5°. The results are expressed in micromoles.

	Substrate utilized	Hydroxy-butyrate formed	O_2 consumed	Citrate formed
Acetate...............................	0	0	105	8.3
Oxalacetate.........................	Not determined	0	128	8.7
Acetoacetate.......................	107	34	128	6.6
Citrate.............................	99	0	101	
Acetate + oxalacetate..............	3	0	133	10.9
Acetoacetate + oxalacetate........	110	33	215	21.8
Acetate + oxalacetate + 0.3 mM BaCl₂.............................	0	0	197	14.7
Acetoacetate + oxalacetate + 0.3 mM BaCl₂.......................	146	42	288	42.7
Acetoacetate + oxalacetate + 0.5 mM BaCl₂.......................	235	80	297	52.0

tate, but citrate is metabolized, though at a lower rate than in slices or minces.

In agreement with Wieland and Rosenthal (3), it was found that the presence of barium ions markedly enhances both the utilization of acetoacetate and the production of citrate. By cutting down the proportion of fluid to around 3 ml. per gm. of wet tissue, a further consistent increase in yield of citrate was observed. Thus, of twelve experiments of 2 hours duration in oxygen, with 10 to 17 gm. of tissue suspended in 46.5 ml. of saline solution containing 0.5 mM of acetoacetate, 1.0 mM of oxalacetate, and 1.5 mM of BaCl₂; the utilization of acetoacetate ranged from 14 to 24 micromoles and the yield of citrate from 7 to 17 micromoles per gm. of wet tissue. Though no stoichiometric relationship was observed, there was

a rough parallelism in the utilization of acetoacetate, yield of citrate, and oxygen consumption.

Isotopically Labeled Citrate from Experiments with Labeled Acetoacetate— To obtain sufficient citric acid for the subsequent isolation and degradation procedures, the products of three experiments made on successive days were combined and worked up together. These experiments are summarized in Table III. Considerable difficulty was experienced at first in separating

TABLE III

Experiments with Carbonyl and Carboxyl C^{13}-Labeled Acetoacetate and Normal Oxalacetate in Rat Kidney Homogenate

Tissue suspended in 46.5 ml. Krebs' phosphate-saline; 2 hours in O_2 at 37.5°.

	Weight of tissue					
	12.5 gm.		16.8 gm.		16.4 gm.	
	mM	C^{13} *excess*	*mM*	C^{13} *excess*	*mM*	C^{13} *excess*
Acetoacetate, start...........	0.413	3.06	0.413	3.06	0.651	3.24
" recovered.......	0.234	3.16	0.042	3.06	0.264	3.08
" utilized.........	0.179		0.371		0.387	
Citrate formed...............	0.107	0.76*	0.198		0.190	
O_2 consumed.................	0.559		1.066		1.043	
CO_2 evolved.................	1.11	0.51	1.45	0.49	1.44	0.65

* Combined citrate from three experiments.

TABLE IV

Distribution of C^{13} in Citric Acid

Values in atom per cent excess.

Method of degradation	Citric acid	Primary carboxyls	Tertiary carboxyl	Non-carboxyl carbons
Oxidation of silver citrate.................	0.76			
Decarboxylation of citric acid.............			0.03	
Decarboxylation of acetonedicarboxylic acid......................................		2.17		
Oxidation with dichromate................				0.00

citric acid from the other substances present in the ether extract, but by following essentially the excellent procedure recently published by Breusch (20), it was possible to isolate in pure form 60 to 70 per cent of the citrate present as the quinidine salt. Because of the high content of extraneous carbon in this salt, it was converted to the silver salt before oxidation to CO_2 for mass spectrometric analysis. The silver citrate contained 0.76 atom per cent excess C^{13}.

The distribution of isotope in the citrate isolated from these experiments is shown in Table IV. All of the excess C^{13} is restricted to the primary car-

boxyls. Their content of 2.17 per cent excess is equivalent to $2.17/3 =$ 0.72 per cent over-all, which is in good agreement with the over-all C^{13} excess of 0.76 per cent found for silver citrate. Assuming that the formation of the isotopic citrate molecule involves the conversion of either a β-carbon or carboxyl carbon of acetoacetate to a primary carboxyl of citrate, $2.17 \times 2 \times 100/6.48 = 70$ per cent of the citrate formed was derived from the isotopic acetoacetate; the remaining 30 per cent must have come from the oxalacetate or the precursors present in the tissue.

The results of all three experiments together show that of the excess C^{13} represented by the total of 0.937 mM of acetoacetate utilized, 19 per cent appeared in the 0.495 mM of citrate formed. Another 19 per cent appeared in the 4.00 mM of respiratory CO_2, 13 per cent was found in the aqueous residue after ether extraction, and 7 per cent in the filtrate after precipitation of the silver salts. About 42 per cent remains unaccounted for.

DISCUSSION

The high content of isotopic carbon in citric acid produced during the aerobic metabolism of C^{13}-labeled acetoacetate leaves no room for further doubt concerning the participation of the tricarboxylic acids in the intermediary processes of fatty acid oxidation. The mechanism by which acetoacetate and oxalacetate produce citrate remains uncertain, however, both with regard to the identity of the reactants and the nature of the primary product.

Both Breusch (19) and Wieland and Rosenthal (3) have postulated a direct coupling of the β-keto acid with oxalacetate, but it seems more likely that the component which condenses with oxalacetate is an acetyl derivative arising by breakdown of acetoacetate. Such a reaction, the reverse of one already established (8), seems highly probable; for example, acetoacetate can give rise to acetate under certain conditions (21) and can yield acetyl groups for acetylation of foreign amino acids (22); moreover, inasmuch as the breakdown of fatty acids to acetyl groups prior to the formation of ketone bodies is already established by isotopic tracer studies the reasonable assumption that this 2-carbon substance, rather than acetoacetate, enters the cycle avoids the necessity of postulating mechanisms involving the intermediary formation of substances whose presence or biological significance remains unproved.

These same considerations apply to the formation of citrate from pyruvate. As previously suggested by Krebs (23) and Martius (24) and discussed recently in great detail by Wood (25), all of the observations on which were based the formulation of the tricarboxylic acid cycle are in complete accord with the hypothesis of an oxidative decarboxylation of pyruvate to an acetyl derivative prior to condensation with oxalacetate. On this basis, the formation of citrate from such substances as acetic (26) and

octanoic acids (27) as well as acetoacetate and pyruvate may be brought
within the scope of a single reaction involving one and the same inter-
mediate.

The observation of Breusch and Keskin (20) that citrate is formed in the
presence of a variety of higher β-keto acids is not in conflict with this view,
since all of the keto acids found active by these investigators could yield
acetyl groups by β cleavage.

Any mechanism for the condensation of acetate with oxalacetate must
account for the following observations: (a) the appearance of C^{13} in the
primary carboxyls of citrate formed during the oxidation of C^{13}-labeled
acetoacetate; (b) the non-equilibration with added citrate of intermediates
of acetate oxidation; (c) the presence of C^{13} predominantly in the δ-carbon
of α-ketoglutarate during oxidation of C^{13}-labeled acetate and acetoacetate;
(d) the presence of isotope exclusively in the carboxyl adjacent to the keto
group of α-ketoglutarate resulting from $C^{13}O_2$ assimilation by pigeon liver
(25).

Because of observations (b), (c), and (d), the direct formation of citrate
as such is excluded; there remain the possibilities either that the primary
condensation product is one of the other tricarboxylic acids or that it is an
unsymmetrical derivative of citric acid.

According to Wood's (25) postulation the primary product is cis-
aconitate, which is presumed to arise by coupling of acetate with the enol
form of oxalacetate.
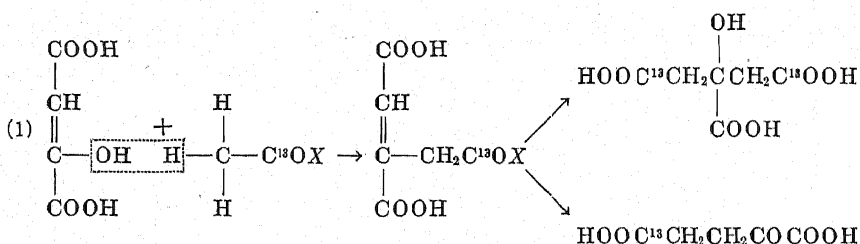
While this scheme is perfectly in accord with all of the isotope data avail-
able at present, there are two reasons for questioning it. First, it is difficult
to conceive of the presence of cis-aconitate as such in systems containing
aconitase without at least partial equilibration with citrate. Second, the
formation of a carbon to carbon linkage in the manner required (intermolec-
ular dehydration) has no counterpart in the field of organic chemistry.
Similar objections can be raised against other mechanisms involving the
formation of isocitrate or oxalosuccinate.

On the other hand, if we assume that a derivative of acetic acid enters the
cycle, the product need not be citrate as such, but an unsymmetrical deriva-

tive, which presumably could undergo a selective transformation to *cis*-aconitate to yield ultimately α-ketoglutarate with an unsymmetrical distribution of C^{13}. A somewhat similar concept has already been suggested by Lynen (28) in connection with the tricarboxylic acid cycle in yeast.

$$(2) \quad \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array} + CH_3C^{13}OX \rightarrow \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ HO-C-CH_2C^{13}OX \\ | \\ COOH \end{array} \rightarrow \begin{array}{c} \overset{OH}{\underset{|}{HOOC^{13}CH_2C-CH_2C^{13}OOH}} \\ | \\ COOH \\ \nearrow \\ HOOC-CH{=}C-CH_2C^{13}OX \\ | \\ COOH \\ \downarrow \\ HOOCCOCH_2CH_2C^{13}OOH \end{array}$$
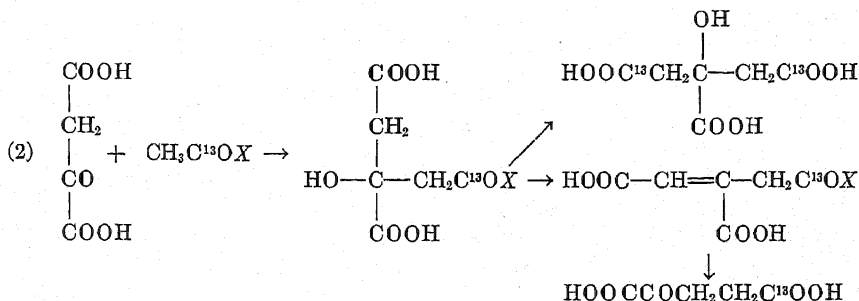
There is a striking similarity in the behavior of "active" acetate and the product of its condensation with oxalacetate. In each case, the substance has not been isolated or identified, but its existence has had to be inferred by indirect methods. This would suggest that the acetyl group is firmly bound to some non-diffusible component, reacting with oxalacetate while in this combination. The fact that foreign amino acids are acetylated may provide a clue to the manner in which such binding occurs. Cohn and du Vigneaud (29) recently emphasized the growing realization that such "detoxication" processes are merely reflections of normal physiological mechanisms. On this basis, it could be assumed that combination of an acetyl group in an amide linkage is a normal step in acetate metabolism.

The fact that substantial quantities of citrate appear only in the presence of relatively large amounts of oxalacetate or its precursors indicates that oxalacetate has some effect other than merely as a component of the reaction. As is shown in Table II, the disappearance of acetoacetate in rat kidney homogenate was about the same in the presence or absence of oxalacetate, but the yield of citrate was over 3 times as high in its presence (*cf.* also Hunter and Leloir (4)). According to mechanism (1), this effect could be attributed to an inhibition by oxalacetate of the further metabolism of *cis*-aconitate, thus allowing time for aconitase action. According to mechanism (2), the effect of oxalacetate could be due either to an inhibition of one of the steps of the cycle or to cleavage of the derivative to form free citrate.

Though there is no clear basis at present for a choice between mechanisms (1) and (2), one difference can be pointed out; namely, mechanism (1) requires the presence of aconitase, whereas mechanism (2) does not. The fact that citrate formation was observed in the washed tissue preparations

of Hunter and Leloir (4) and Lehninger (27), from which a considerable part of the soluble aconitase must have been removed, may be cited in favor of mechanism (2).   The aconitase activity of tissue preparations which yield citrate is now under investigation.

The authors wish to express their appreciation to the Sun Oil Company for its interest in and support of this work.

SUMMARY

When C^{13}-labeled acetate was oxidized by rat liver slices and rat kidney slices or minces in the presence of added cis-aconitate or citrate, the recovered citrate had no significant excess of C^{13}, indicating that intermediates of acetate metabolism do not come into equilibrium with added tricarboxylic acids.

However, when oxalacetate and acetoacetate, labeled with 6.48 atom per cent excess C^{13} in the β and carboxyl positions, were incubated aerobically with rat kidney homogenate, the citrate formed had a C^{13} excess of 2.17 per cent in the primary carboxyl carbons and a normal C^{13} content in the other carbons.

The formation of isotopic citrate is taken as positive evidence for the participation of the tricarboxylic acid cycle in fatty acid metabolism.   Possible mechanisms are discussed.

BIBLIOGRAPHY

1. Weinhouse, S., Medes, G., Floyd, N. F., and Noda, L., *J. Biol. Chem.*, **161**, 745 (1945).
2. Breusch, F. L., *Science*, **97**, 490 (1943).
3. Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).
4. Hunter, F. E., and Leloir, L. F., *J. Biol. Chem.*, **159**, 295 (1945).
5. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **159**, 695 (1945).
6. Weil-Malherbe, H., *Nature*, **153**, 435 (1944).
7. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **39**, 408 (1945).
8. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **158**, 411 (1945).
9. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 415 (1929).
10. Schneider, W. C., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques, Minneapolis, 187 (1945).
11. Malachowski, R., and Maslowski, M., *Ber. chem. Ges.*, **61**, 2521 (1928).
12. Medes, G., Floyd, N. F., and Weinhouse, S., *J. Biol. Chem.*, **162**, 1 (1946).
13. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
14. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
15. Pucher, G. W., *J. Biol. Chem.*, **153**, 133 (1944).
16. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).
17. Organic syntheses, New York, coll. **1**, 10 (1932).

18. Bloch, K., Borek, E., and Rittenberg, D., *J. Biol. Chem.*, **162**, 441 (1946).
19. Breusch, F. L., *Enzymologia*, **11**, 169 (1944).
20. Breusch, F. L., and Keskin, H., *Enzymologia*, **11**, 243 (1944).
21. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
22. Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).
23. Krebs, H. A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3**, 232 (1943).
24. Martius, K., *Z. physiol. Chem.*, **279**, 96 (1943).
25. Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).
26. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).
27. Lehninger, A. L., *J. Biol. Chem.*, **161**, 413 (1945).
28. Lynen, F., *Ann. Chem.*, **554**, 40 (1943).
29. Cohn, M., and du Vigneaud, V., Paper presented before the biological division of the American Chemical Society, Atlantic City, April 10, 1946.

THE DETERMINATION OF SERUM AMYLASE, WITH PARTICULAR REFERENCE TO THE USE OF β-AMYLOSE AS THE SUBSTRATE

By MARIE A. ANDERSCH

(*From the Department of Medicine, Subdepartment of Clinical Pathology, University of Maryland, School of Medicine, and Biochemistry Laboratory, University Hospital, Baltimore*)

The estimation of serum amylase by the saccharimetric method is based upon the digestion of a starch solution with serum followed by the determination of the reducing substances produced. Differences in numerical values obtained by modifications of the basic method may result from the use of various types of starch (1–3), from differences in the pH of the digestion mixture (1–3), and from variations in the sensitivity of the procedure ultimately used for the determination of the end-products of the reaction. Many of the usual alkaline copper reagents are not readily reduced by maltose. The starch solutions used as the substrate frequently hinder the smooth performance of the test. They tend to give cloudy filtrates following the precipitation of the plasma proteins and thereby interfere with the use of the photoelectric colorimeter for the estimation of the reducing substances. Starch also retrogrades and comes out of solution so that the substrate must be renewed frequently. Myers *et al.* (3) have overcome some of these difficulties by using a buffered starch solution and determining the reducing substances by the reduction of picric acid. The products of the digestion reduce this substance to a greater degree than they do either the Folin-Wu or the Benedict reagents (4). Myers *et al.* have, however, continued to use a starch substrate. It would seem desirable to use some substrate which would (1) be in a stable form so that it would not have to be prepared frequently, (2) would give clear filtrates with the usual protein precipitants, and (3) would have a low blank reducing value. It was thought that some fraction of starch might meet these requirements.

EXPERIMENTAL

β-Amylose As Substrate—Corn and potato starch consist of at least three components (5), each of which may vary in per cent in various starches (6). One fraction, β-amylose, can be preferentially adsorbed by cellulose (7) to the extent of 1.7 per cent (8). The β-amylose from potato starch differs from that obtained from corn in that it is relatively more soluble and tends

to retrograde more slowly when brought into solution (5). It was therefore considered as a possible substrate for amylase, either in its adsorbed state or in solution.

The β-amylose can be prepared in the following manner: Wash 100 gm. of absorbent cotton three times with distilled water and squeeze out the excess fluid. Prepare 1000 ml. of a 1 per cent potato starch in the usual manner and cool. The solution is then poured over the cotton and allowed to stand for several hours. The cotton should be agitated occasionally so that it becomes thoroughly saturated. Wash the cotton in cold running water until the wash water no longer gives a reaction for starch with iodine. The water is squeezed out and 250 ml. of absolute methyl alcohol are poured over it in order to dehydrate the β-amylose. The alcohol is removed and a second portion added. The methanol is then removed as completely as possible and the β-amylose-treated cotton dried with the aid of an electric fan. If sheet cotton is kept intact, a given weight may be estimated by measuring it off in sq. in. after the weight per sq. in. has been ascertained.

β-Amylose appeared to be stable in this form and tests showed that it could be directly hydrolyzed from the cotton by amylase.[1] It was then necessary to determine whether the reducing substances produced under standard conditions were proportional to the relative concentration of diastase.

Proportionality between Enzyme Concentration and Reducing Substances —1 gm. samples of β-amylose-treated cotton were digested in the presence of phosphate buffer of pH 7.0 and adequate sodium chloride for maximum activity (9) with a mixture of saliva and pancreatin. The maximum reducing substances produced in two samples in 2 hours were 16 and 18 mg. of glucose respectively. A series of tests was then run with dilutions of the enzyme solution in order to determine the relationship between relative enzyme concentration and the production of reducing substances following 15 minutes incubation with β-amylose-treated cotton and by the standard Somogyi technique (Fig. 1) with a 1.5 per cent starch substrate and an incubation period of 30 minutes at 40° (1). It is seen that the proportionality between relative enzyme concentration and reducing substance produced holds very well up to 1000 mg. of reducing sugar per 100 ml. of enzyme solution when the β-amylose-treated cotton is used and up to around 700 mg. with the Somogyi technique.

The values obtained for serum amylase depend not only upon the substrate but also upon several interrelated factors, all of which must be controlled if reproducible results are to be obtained.

---

[1] β-Amylose-treated cotton has been used over several months without apparent change in activity.

pH and Salt Concentration—The concentration of sodium chloride must be 0.01 M in the substrate before the enzyme activity no longer increases with salt concentration. At salt concentrations of this value and above, the optimum pH for pancreatic amylase is approximately 7.1 (9). With a dilution of 0.5 ml. of serum to 3 ml., which we proposed to use for the reaction, the sodium chloride would be close to the minimum concentration. Therefore 0.5 ml. of 5.8 per cent sodium chloride was added in order to insure a sufficient quantity for 0.5 ml. samples as well as for smaller samples which would have to be used if very high amylase values were encountered.
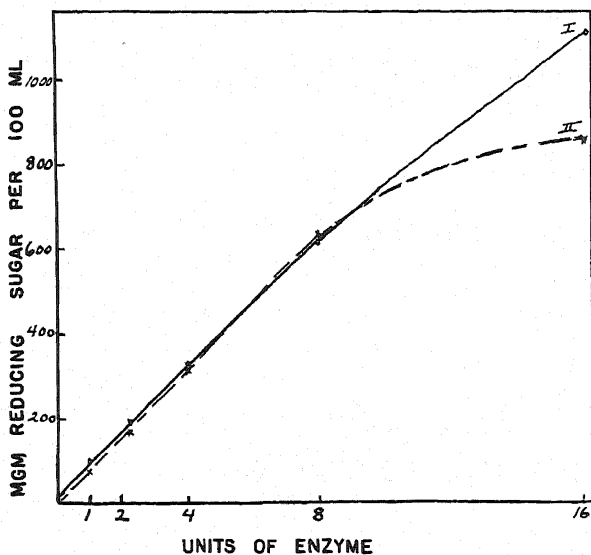
FIG. 1. Curve I, mg. of reducing substances produced per 100 ml. of enzyme solution in 15 minutes with a β-amylose-treated cotton substrate; Curve II, mg. of reducing substances produced per 100 ml. of enzyme solution in 30 minutes with the Somogyi method.

Myers *et al.* (3) have pointed out the necessity of using a buffered substrate and have called attention to the deficiency of the Somogyi substrate in this regard. Their observation that the substrate reaches a pH of above 8.0 with the loss of carbon dioxide has been partially confirmed, but the highest pH which we found was 7.8. This, however, is outside the optimum pH for the enzyme (9). That pH is an important factor in the actual determination was demonstrated by the results of seven analyses made by the original Somogyi technique and by modifying this procedure by the addition of a comparable amount of sodium chloride to the substrate and substitution of 2 ml. of buffer, pH 6.8, for the 2 ml. of acid

sodium chloride. The average of the seven determinations without buffer was 77 mg., while that with buffer was 142 mg. per 100 ml. of serum.

Time and Temperature of Incubation—The time and temperature of incubation appear to be arbitrarily chosen for different modifications. A temperature of 37.5° ± 0.5° was selected for convenience, since this conforms to the usual temperature at which water baths and incubators are set. The digestion was allowed to proceed for both 15 and 30 minute periods and the 15 minute period was finally selected because the values for normal sera conformed with those found by the Somogyi method for a 30 minute incubation period.

Precipitation of Proteins and Determinations of Reducing Substances— The precipitation of the serum protein with barium hydroxide and zinc sulfate (10), followed by the removal of the unused substrate from the filtrate, serves to stop the reaction. Filtrates kept at room temperature for 2 hours and in the refrigerator for 24 hours show no increase in reducing substances.

The Somogyi high alkaline reagent is sensitive to maltose and other slow reacting sugars (11). The Nelson color reagent used in conjunction with this reagent produces a color of higher intensity and greater stability than is produced by other color reagents. It is therefore possible to increase the dilution of the filtrate without sacrificing accuracy. This eliminates the necessity for repetition of the sugar determination on smaller aliquots when high serum amylase values are encountered.

Procedure

Reagents—

1. Phosphate buffer, pH 6.8. To 250 ml. of 0.2 M potassium dihydrogen phosphate are added 118 ml. of 0.2 M sodium hydroxide, and the mixture diluted to 1000 ml.

2. Somogyi high alkaline copper reagent (11).

3. Nelson color reagent (12).

4. β-Amylose adsorbed on cotton.

5. Sodium chloride, 5.7 per cent.

6. Zinc sulfate (7 moles of water), 5.0 per cent (10).

7. Barium hydroxide, 0.3 N. (The zinc sulfate and the barium hydroxide should titrate volume for volume with phenolphthalein as the indicator (10).)

Determination—To 2 ml. of the phosphate buffer in a wide mouthed bottle or flask are added 0.5 ml. of 5.7 per cent salt solution and 0.5 ml. of serum. This is allowed to reach equilibrium in the water bath and 1 gm. of β-amylose-treated cotton is dropped in and stirred with a glass rod until the fluid is taken up by, and distributed throughout the cotton. The

flask is corked and incubated for 15 (or 30) minutes. 1 ml. of 0.3 N barium hydroxide and 15 ml. of water are added, followed by 1 ml. of zinc sulfate. The mixture is stirred thoroughly and the supernatant liquid is decanted into a centrifuge tube and centrifuged for 10 minutes. A 2 ml. aliquot of the clear supernatant fluid is measured into a tube graduated at 25 ml. and the sugar determined according to the procedure of Somogyi (11). A serum sugar is determined at the same time on a 1:10 filtrate prepared by adding 5 ml. of water, 2 ml. of barium hydroxide, and 2 ml. of zinc sulfate to 1 ml. of serum and centrifuging. The serum sugar is subtracted from the total reducing substances. It should be noted that the amylase determination is made on a 1:40 dilution and the serum sugar on a 1:10. The substrate blank runs consistently between 2 and 5 mg. for each batch of substrate and may be either determined once for each preparation of substrate or may be omitted entirely.

Normal Values

Three series of determinations were made on a group of normal individuals ranging from twenty-one to 50 years of age. The Somogyi method for amylase was used as well as determinations with the β-amylose-treated cotton substrate, with incubation of the latter at both 15 and 30 minutes.

Forty-three determinations with the Somogyi method resulted in an average value of 58 mg. per 100 ml. of serum, with 90 per cent falling within the range from 30 to 135 mg. The 15 minute incubation of 55 sera with the β-amylose-treated cotton gave an average of 73 mg., with 90 per cent falling between 40 and 145 mg. Following 30 minute incubation, the average for forty-seven sera was 156 mg., with 90 per cent falling between 95 and 250 mg. With the exception of one serum which gave high values with each method, those falling outside the range given were consistently lower. The 15 minute incubation period under the conditions used gave values which were within the numerical range of normal values of 40 to 175 mg. obtained by Lewison (13) on the bloods of a selected group of patients by the Somogyi technique. This fortuitous result makes it unnecessary to change markedly the concept of normal values from those established by Somogyi. The lower limit of normal need only be lowered from the original value of 80 to 40 mg. The normal range for the Somogyi technique would likewise probably be lowered to 40 mg. in view of the values which were obtained by Lewison on a large series of bloods.

SUMMARY

1. β-Amylose adsorbed from potato starch on cotton has been shown to serve as a stable substrate for the determination of serum amylase.

2. Satisfactory correlation between enzyme concentration and reducing substances produced is obtained up to about 1000 mg. per 100 ml. of serum.

3. This substrate does not interfere with the photoelectric determination of reducing sugars.

4. 55 sera which were incubated for 15 minutes with this substrate gave an average of 73 mg. per 100 ml. of serum, with 90 per cent falling between 40 and 145 mg.

5. Following an incubation period of 30 minutes, the average when this substrate was used for forty-seven sera was 156 mg. with 90 per cent falling between 95 and 250 mg.

6. Forty-three determinations by the Somogyi method gave an average of 58 mg. with 90 per cent falling between 30 and 135 mg.

BIBLIOGRAPHY

1. Somogyi, M., *J. Biol. Chem.*, **125**, 399 (1938).
2. Dozzi, D. L., *J. Lab. and Clin. Med.*, **25**, 1303 (1940).
3. Myers, V. C., Free, A. H., and Rosinski, E. E., *J. Biol. Chem.*, **154**, 39 (1944).
4. Myers, V. C., and Reid, E., *J. Biol. Chem.*, **99**, 595 (1932–33).
5. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957 (1942).
6. Kerr, R. W., and Severson, G. M., *J. Am. Chem. Soc.*, **65**, 193 (1943).
7. Pacsu, E., and Mullen, J. W., 2nd, *J. Am. Chem. Soc.*, **63**, 1168 (1941).
8. Samec, M., *Ber. chem. Ges.*, **73** A, 85 (1940).
9. Sherman, H. C., Caldwell, M. L., and Adams, M., *J. Am. Chem. Soc.*, **50**, 2529 (1928).
10. Somogyi, M., *J. Biol. Chem.*, **160**, 69 (1945).
11. Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945).
12. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
13. Lewison, E. F., *Surg., Gynec., and Obst.*, **72**, 202 (1941).

INFLUENCE OF CHOLINE AND METHIONINE ON PHOSPHO-LIPIDE ACTIVITY AND TOTAL LIPIDE CONTENT OF LIVERS OF YOUNG WHITE RATS*

By MARJORIE G. HORNING† AND H. C. ECKSTEIN

(*From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*)

According to recent reviews (1, 2) the lipotropic action of choline, methionine, and allied substances can best be explained by an acceleration of phospholipide turnover in the liver. Some of the evidence upon which this view is based was supplied by recent studies by Perlman and coworkers with radioactive phosphorus (3, 4). In experiments with rats that had ingested a low protein-high fat diet, this group from California clearly demonstrated that more of this radioactive element found its way to the phospholipide fraction of the livers of rats receiving supplements of choline or methionine than of those to whom the unsupplemented diet alone was fed. These findings led to the conclusion that phospholipide metabolism in the liver had been speeded up by these supplements and that this increase in activity was associated with lipotropic action. Unfortunately no data on total lipide content of the livers were included, and the desired proof of the existence of a relationship between this apparent increased activity of liver phospholipides and the decrease in liver lipide content was lacking. The present investigation was planned with this in mind. A study was made of the effect of choline and methionine on total lipide content, the phospholipide content, and the phospholipide activity of young male rats that had ingested a diet low in protein and high in fat.

The experimental procedure consisted of placing young male rats (weight 70 to 100 gm.) on a diet containing 5 per cent casein, 2 per cent agar, 5 per cent salt mixture (5), 48 per cent glucose, and 40 per cent fat (lard, elaidin, or a mixture of equal parts of lard and elaidin) for periods ranging from 10 to 28 days. Each rat received 1 tablet of dry yeast (500 mg.) and 2 drops of cod liver oil daily. In approximately half of the experiments, the diet was fed *ad libitum;* in the remaining studies, the system of paired feeding of litter mates was adopted. 8 hours prior

to the termination of the periods, choline or methionine was administered by stomach tube and radioactive phosphorus,[1] in the form of sodium phosphate (pH 8), subcutaneously. This procedure was chosen because of the demonstration by Perlman and Chaikoff (3) that the maximum phospholipide turnover, as determined with radioactive phosphorus, occurs in the liver between the 3rd and 8th hours following the administration of choline. At the end of this interval, the animal was stunned by a blow at the base of the brain, the head was severed, and bleeding was allowed to proceed freely. The liver was removed, frozen in solid carbon dioxide, weighed, and pulverized in a metal crusher (6). Total lipides were extracted with absolute alcohol and ether in the manner previously described (7). Phospholipides were precipitated in the usual manner with acetone and alcoholic magnesium chloride, redissolved in petroleum ether, and reprecipitated with acetone. Aliquots of the purified product were oxidized with sulfuric acid and superoxol (30 per cent hydrogen peroxide), and these solutions were used for phosphorus determination by the Fiske-Subbarow method (8), as modified by us to permit the use of the Evelyn photoelectric colorimeter. In making the calculation for phospholipides it was assumed that these contained 4 per cent phosphorus. A separate portion of the acid digest was made alkaline with sodium hydroxide (pH 10) and evaporated to dryness. The radioactivity of this aliquot was determined with the Geiger-Müller counter.

Before an evaluation of our experimental data is made, it must be made clear that the procedure adopted to demonstrate lipotropic action differs fundamentally from the orthodox method in which compounds to be tested are included in the diet for a considerable length of time (2 or 3 weeks). In our experiments, the diet was fed during periods varying from 10 days to 4 weeks and the lipotropic substance administered only once (8 hours prior to the end of the experimental period). In addition, it is recognized that overlapping between values for the experimental and control values are common findings in investigations of the phenomenon of fatty livers. Some investigators (9, 10) have recently adopted the policy of pooling livers of different groups of animals for analysis, but even then inconsistencies in results appear. We therefore anticipated overlapping of data and no great differences between those of the experimental and control rats.

Table I summarizes the effect of the administration of methionine to young male rats receiving the stock diet *ad libitum*. In these experiments the dietary fat consisted of lard. A comparison of the data for the experimental and control animals shows that lipotropic action can take place

---

[1] The radioactive phosphorus used in this investigation was supplied through the courtesy of Dr. J. M. Cork of the Department of Physics, University of Michigan.

TABLE I

Influence of Methionine on Total Lipide and Phospholipide Content and P^{32} Activity of Phospholipides of Livers of Young Male Rats on High Fat Diet

dl-Methionine (Merck) was given as a single dose (250 mg.) by stomach tube 8 hours prior to the end of the experimental periods which were 4 weeks in duration in Series 1, 2, and 3, and 3 weeks in Series 4. P^{32} activity refers to per cent of administered P^{32} recovered in liver phospholipides. Total lipides are expressed as per cent of fresh liver, phospholipides as per cent of fresh, fat-free liver.

Series No.	Rat No.	Methionine added			Rat No.	No methionine added		
		Total lipides	Phospho-lipides	P^{32} activity		Total lipides	Phospho-lipides	P^{32} activity
		per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
1	91	29.4	3.9	3.3	100	42.5	3.0	2.2
	92	31.3	3.9	3.0	101	32.0	2.6	2.7
	93	29.2	3.2	2.6	102	49.0	3.3	1.7
	94	24.8	3.3	3.0				
Average.........		28.7	3.6	3.0		41.2	3.0	2.2
2	76	31.1	3.6	12.6	86	28.2	2.6	2.9
	77	20.2	3.0	17.1	87	25.7	2.9	2.8
	78	28.1	3.1	4.5	88	40.0	3.3	3.0
	79	27.3	3.4	3.7	89	28.4	2.5	2.2
	80	26.6	3.4	3.5	90	30.8	2.7	2.0
Average.........		26.7	3.3	8.3		30.6	2.8	2.6
3	61	35.3	4.1	2.7	65	37.1	3.5	3.1
	63	18.2	3.6	2.7	70	39.9	3.7	2.7
	66	30.4	4.4	2.7	73	35.2	3.5	2.9
	67	42.7	3.5	2.7	74	31.2	3.6	3.0
	69	24.5	3.5	2.8	75	27.0	3.6	2.9
Average.........		30.2	3.8	2.7		34.1	3.6	2.9
4	123	20.8	3.6	3.2	119	36.2	3.0	2.9
	125	39.5	3.6	3.5	127	32.5	3.6	3.4
	126	33.5	3.5	3.6	130	16.9	3.0	3.5
	128	26.8	3.9	3.8	131	29.5	3.3	3.1
	129	38.2	3.6	4.1	132	38.1	3.4	3.1
Average.........		31.8	3.6	3.6		30.6	3.3	3.2
Average all rats..		29.4	3.6	4.5		33.3	3.2	2.8

within 8 hours after the feeding of methionine. In Series 1, the values for total liver lipide content for all of the experimental animals are signifi-

cantly lower than, those for the control rats. In the remaining series, lipotropic action is again in evidence but overlapping of the values for liver lipide concentration is common. These inconsistencies may be due to the fact that no attempt was made to control the food intake. Table I further shows that, in the majority of the cases, the administration of methionine is followed by a slight increase in liver phospholipide content, but such increases are not necessarily accompanied by lipotropic action. The remaining data in Table I demonstrate that the phospholipide activity, as determined with radioactive phosphorus, of the experimental rats is usually higher than that of the controls. In Series 1, the average increase in activity (36 per cent) following the oral administration of supplementary methionine compares well with the increments in activity of 32, 33, and 41 per cent reported by Perlman, Stillman, and Chaikoff (4) for three series of rats receiving this acid as a supplement. In Series 2 and 4 the values for phospholipide activity of the experimental animals again exceed those of the control animals, but these increases are not of necessity associated with lipotropic action. This lack in parallelism is further illustrated in Series 3 and 4. In the former, lipotropic action occurred in Rats 63 and 69 even though the amount of radioactive phosphorus that migrated to the phospholipide in the livers of these two animals did not exceed the amount recovered in the liver phospholipides of the remaining animals of the series. In Series 4, the average value for total liver lipide concentration in the experimental animals was higher than that of the controls in spite of the fact that the phospholipide activity increased as a result of the administration of the lipotropic agent. Evidently an increase in phospholipide turnover, as determined with radioactive phosphorus, is not necessarily associated with the removal of fat acids from the liver.

In the experiments grouped in Table II, the system of paired feeding of male litter mates was adopted. Elaidin, prepared according to Sinclair (11), was introduced in the diets with the hope of further labeling the phospholipides of the liver. This proved to be unsuccessful. In assaying the liver phospholipide fraction for this acid a slight modification of the Sinclair procedure was found desirable. The change consisted primarily of removing the brown contaminant of the insoluble lead salt fraction by passing the hot alcoholic solution through a sintered glass Hirsch funnel equipped with a steam-heated outer jacket instead of attempting to eliminate the impurity by centrifugation or decantation, as recommended by Sinclair (12). In addition a modified Hanus method for the determination of the iodine number was substituted for the Rosenmund-Kuhnhenn procedure employed by Sinclair. The exact details of our modified

<div align="center">TABLE II</div>

Influence of Choline on Total Lipide and Phospholipide Content and Activity
of Phospholipides of Livers of Young Male Rats on High Fat Diet

Choline chloride (Eastman) was given as a single dose (30 mg.) by stomach tube 8 hours prior to the end of the experimental periods which were 3 weeks in duration in Series 5, 6, and 7, and 10 days in Series 8. In Series 5, 6, and 7 the lard (40 per cent of the stock diet) was replaced by an equivalent amount of elaidin during the last 2 days. In Series 8 the dietary fat (40 per cent) consisted of an equal mixture of lard and elaidin during the whole period. Total lipides are expressed as per cent of fresh liver, phospholipides as per cent of fresh, fat-free liver. Phospholipide activity refers to per cent of administered radioactive phosphorus recovered in liver phospholipides. Paired feeding was followed throughout these experiments.

Series No.	Pair No.	Choline chloride added			No choline chloride added		
		Total lipides	Phospho-lipides	Phospho-lipide activity	Total lipides	Phospho-lipides	Phospho-lipide activity
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
5	2	10.5	3.5	2.2	22.2	3.3	1.9
	3	11.8	3.8	1.9	18.0	3.7	1.5
	4	26.5	4.1	3.5	19.7	3.2	1.3
	5	10.5	3.9	2.7	22.0	3.7	2.5
Average		14.8	3.8	2.6	20.5	3.5	1.8
6	7	8.5	3.5	3.6	8.6	2.9	2.6
	8	9.6	3.8	3.7	9.7	3.1	3.1
	9	6.0	3.2	4.0	9.3	3.7	2.7
	10	10.0	3.2	3.4	10.6	2.9	1.6
	11	7.3	3.8	3.5	6.9	3.2	2.9
	12	6.8	3.3	3.5	8.8	3.1	2.6
Average		8.0	3.5	3.6	9.0	3.2	2.6
7	15	10.7	3.6	2.9	15.7	3.0	2.1
	16	14.7	3.4	3.8	19.9	3.6	2.3
	17	11.1	3.6	3.6	26.9	2.8	1.9
	18	14.1	3.3	6.2	15.6	3.1	2.3
	19	13.0	3.1	3.7	19.7	2.8	2.5
	20	9.8	3.6	4.1	12.7	3.0	1.9
Average		12.2	3.4	4.1	18.4	3.1	2.2
8	24	7.8	4.0	4.8	9.9	4.1	3.0
	25	12.1	4.2	5.2	10.5	3.5	3.0
	26	8.9	3.7	3.8	11.6	3.9	3.6
	27	11.5	3.4	4.1	19.6	3.5	3.0

TABLE II—*Concluded*

Series No.	Pair No.	Chlorine chloride added			No choline chloride added		
		Total lipides	Phospho-lipides	Phospho-lipide activity	Total lipides	Phospho-lipides	Phospho-lipide activity
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
8—continued	28	13.6	3.6	5.4	11.3	3.2	3.2
	29	11.5	3.3	4.3	13.6	3.3	2.8
	30	14.0	3.0	3.7	17.3	3.1	3.0
	31	9.2	3.3	3.8	8.5	3.2	3.0
	32	14.2	3.7	3.6	11.1	4.1	3.6
Average........		11.5	3.5	3.8	12.6	3.4	3.1
Average all rats..........		11.5	3.6	3.8	14.4	3.3	2.6

procedure are described elsewhere (13). With these modifications small amounts of elaidic acid added to liver phospholipide fat acids could be quantitatively estimated.

This is illustrated in Table III. On the basis of the iodine numbers of Samples D-1, D-2, and E the iodine value of Sample F should have been 42.9. This is in agreement with the actual value of 43.7 obtained for Sample F. Similarly the calculated value of 117 for the weight of the solid fat acid fraction of Sample F is in good agreement with the recorded value of 113. The calculation was based on the results for weights of solid fat acids of Samples D-1, D-2, and E. However, no elaidic acid was

TABLE III

Effect of Added Elaidic Acid on Iodine Number and Solid Fat Acids of Phospholipides of Rat Livers

Sample No.	Total fat acids	Solid fat acids	Iodine No.
	mg.	*mg.*	
C-1	139	54	13.7
C-2	139	54	12.7
D-1	126	50	11.4
D-2	126	50	13.3
E	134	134	63.0
F	192	113	43.7

Samples C-1, D-2, D-1, and D-2 consisted of the combined fatty acids obtained by saponifying the phospholipides of the livers of rats on diets devoid of elaidin. Sample E represents the fatty acids liberated by the saponification of elaidin employed in this investigation. Sample F was a mixture of 125 mg. of the acids analyzed in Samples D-1 and D-2 and 67 mg. of the elaidic acid used in Sample E.

detected in the phospholipide fraction of the livers of rats that had ingested considerable amounts of elaidin.

We were led to this view from the data summarized in Table IV, wherein it is shown that the differences between the iodine numbers of the solid fat acid fraction derived from the phospholipides of rats fed lard and those ingesting lard plus elaidin were not significant. This finding is in contrast to that of Sinclair (12) who reported decided increases in the iodine value for this fraction of the phospholipides of rats fed elaidin under circumstances comparable with ours. This may be due to differences in analytical procedure. In our method a brown contaminating material was removed by filtration through a steam-heated sintered glass Hirsch filter, as previously described. The complete elimination of the contaminant was not possible when we applied the procedure outlined by Sinclair (12). During the course of our studies a considerable amount of this brown contaminant was accumulated. Analysis showed the presence of lead (approximately 45 per cent). Upon acidification with acetic or hydrochloric acid, a product soluble in ethyl ether was liberated. This acidic material combined with halogen and had an iodine number of 60.

It is apparent from the values for total lipide content in Table II that, as in the case of methionine, the lipotropic action of choline can be demonstrated during these short periods. This is strikingly illustrated in Series 5 and 7. In the latter case this action occurs without exception and in the former in three of the four pairs. The lipotropic action is not marked in Series 6 and 8 and overlapping of the values for total lipide concentration is quite evident. The difference in response is probably due to the fact that the average total lipide content in Series 6 and 8 is comparatively low. It has been our experience and also that of Channon, Mills, and Platt (9) that it becomes increasingly more difficult to demonstrate lipotropic action with methionine or choline in experiments where the "fat" content of the livers of the control rats is comparatively low. In this connection it is obvious from Table II that the most striking illustration of lipotropic action in Series 8 is exhibited by Pairs 27 and 30 in which the total lipide content of the controls is of the same order of magnitude as that of the controls of Series 5 and 7. A further study of the data in Table II indicates that these higher values for liver lipide content are usually accompanied by values in liver phospholipide content that are lower than 3.5 per cent. Our average value for the phospholipide content of the livers of young male rats that had been on the regular Rockland rat diet was 3.5 per cent. This fall in the liver phospholipide concentration as a result of the ingestion of a lipogenic diet is in line with the recent report by Artom and Fishman (14). In addition Table II shows that, in a majority of the pairs, the phospholipide content of the liver of the rat receiving choline is somewhat

TABLE IV

Influence of Dietary Elaidin on Iodine Number of Solid Fat Acids in Phospholipides of Rat Livers

Experiment No.	Fat in diet*	No. of animals	Total phospholipide fat acids	Solid fat acids in phospholipides	Iodine No.
			mg.	mg.	
1†	40% lard	2	131	29	12.1
	40% " + elaidin	4	223	70	13.0
	40% " + " + choline	4	289	73	27.0
2†	40% "	2	144	31	3.4
	40% "	2	144	31	3.2
	40% " + elaidin	3	254	51	7.5
	40% " + " + choline	3	298	62	3.9
3‡	40% "	3	283	35	1.6
	40% "	3	287	44	5.3
	20% " + 20% elaidin	3	330	55	1.6
	20% " + 20% "	3	331	48	6.1
	20% " + 20% " + choline	3	307	47	4.0
	20% " + 20% " + "	3	341	49	3.5
	20% " + 20% " + "	3	350	53	11.5
4§	40% "	3	133	42	7.3
	40% "	2	115	29	10.6
	20% " + 20% "	3	197	41	10.1
	20% " + 20% "	3	200	41	13.9
	20% " + 20% "	3	221	49	9.8
	20% " + 20% " + choline	3	267	62	11.1
	20% " + 20% " + "	3	237	71	14.9
	20% " + 20% " + "	3	256	97	8.5
5‖	40% "	3	267	74	7.3
	40% "	2	173	37	13.4
	20% " + 20% "	3	282	58	12.2
	20% " + 20% "	3	328	58	11.1
	20% " + 20% "	3	271	51	11.5
	20% " + 20% " + methionine	3	364	66	16.0
	20% " + 20% " + "	3	359	64	12.8

* Paired feeding was followed throughout these experiments.

† In Series 1 and 2 the animals were maintained on the high fat diet for 21 days. During the last 2 days of this period elaidin was substituted for the fat of the diet in the cases indicated, and in addition elaidin was administered by medicine dropper four times daily at 4 hour intervals during these 2 days. Each animal consumed approximately 10 gm. of elaidin. Choline chloride (30 mg.) was administered by stomach tube 8 hours before the conclusion of the experiment.

‡ These animals were maintained on a high fat diet for 10 days. In the cases indicated, the diet contained 20 per cent lard and 20 per cent elaidin. The consumption of elaidin was approximately 17 gm. per animal. Choline chloride (30 mg.) was administered by stomach tube 8 hours before the conclusion of the experiments.

TABLE IV—*Concluded*

§ These animals were maintained on a high fat diet for 19 days. In the cases indicated, the diet contained 20 per cent lard and 20 per cent elaidin. The consumption of elaidin was approximately 30 gm. per animal. Choline chloride (30 mg.) was administered by stomach tube 8 hours before the conclusion of the experiments.

‖ These animals were maintained on a high fat diet for 21 days. In the cases indicated, the diet contained 20 per cent lard and 20 per cent elaidin. The consumption of elaidin was approximately 33 gm. per animal. Methionine (250 mg.) was administered by stomach tube 8 hours before the conclusion of the experiments.

larger than that of his control mate. The differences are, however, small and may not be related to the phenomenon of fatty livers, since increases in liver phospholipide content are observed in pairs in which no lipotropic action was demonstrated. The influence of choline on phospholipide activity is well illustrated in Table II by the fact that in twenty-four of the twenty-five pairs the administration of choline resulted in the migration of greater amounts of radioactive phosphorus to the liver phospholipides of the experimental rats. The average increase in activity (46 per cent) resulting from the injection of choline compares well with a corresponding increment of 45 per cent observed by Perlman and Chaikoff (3) in similar experiments terminated 6 hours following the administration of choline. This increase in activity is not necessarily indicative of lipotropic action, since, as is shown from our data in Table II, it was accompanied by a fall in liver lipide content in only two-thirds of the experimental animals.

As has already been pointed out, we are entirely in agreement with Perlman and coworkers (3, 4) who observed that the administration of choline or methionine to rats on a lipogenic diet results in the transport of additional radioactive phosphorus to the phospholipide fraction of the liver. We have, in addition, shown that the lipotropic action of methionine or choline can be demonstrated within 8 hours after the oral administration of these substances. The average fall in liver lipide content was appreciably greater following the feeding of choline. This quantitative difference in response in these shorter experiments might be ascribed to the probability first suggested by du Vigneaud *et al.* (15) that methionine owes its lipotropic activity to a transmethylation reaction whereby its methyl group contributes to the synthesis of choline. If this is necessary it might be expected that during our short term experiments the response to administered choline would be more rapid. During these 8 hours, there is a general tendency for a small increase in liver phospholipide concentration when choline or methionine is fed. This increase in concentration, however, is not always accompanied by a fall in liver lipide content, and hence may not be related to the phenomenon of fatty livers. In the same manner a lack of parallelism exists between lipotropic action

and increase in phospholipide turnover as determined with radioactive phosphorus. In some of the experiments with methionine, lipotropic action had evidently taken place without any apparent increase in phospholipide activity; in others the feeding of the amino acid accelerated the transfer of radioactive phosphorus without a fall in liver lipide content. This lack in parallelism is also apparent when choline is given. Evidently an increase in phospholipide turnover as determined with the radioactive element does not of necessity indicate an increase in fat metabolism in the liver.

SUMMARY

1. The lipotropic action of methionine and choline can be demonstrated 8 hours after the oral administration of these methylated products to young male rats that had previously ingested a lipogenic diet for periods varying from 10 days to 4 weeks.

2. The oral administration of methionine or choline is usually followed by an increased transport of radioactive phosphorus to the phospholipide fraction of the liver as well as a slight increase in concentration of liver phospholipides. These increases are not always accompanied by a fall in liver lipide content and may not be related to lipotropic action.

3. Under the conditions obtaining in our experiments it was not possible to detect elaidic acid in the phospholipides of the livers of young male rats that had ingested considerable amounts of elaidin.

BIBLIOGRAPHY

1. Best, C. H., and Lucas, C. C., Vitamins and hormones, New York, 1, 1 (1943).
2. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
3. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **127**, 211 (1939).
4. Perlman, I., Stillman, N., and Chaikoff, I. L., *J. Biol. Chem.*, **133**, 651 (1940).
5. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
6. Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., *J. Biol. Chem.*, **104**, 149 (1934).
7. Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, **121**, 479 (1937).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Channon, H. J., Mills, G. T., and Platt, A. P., *Biochem. J.*, **37**, 483 (1943).
10. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275 (1943).
11. Sinclair, R. G., *J. Biol. Chem.*, **111**, 515 (1935).
12. Sinclair, R. G., *J. Biol. Chem.*, **134**, 71 (1940).
13. Groothuis, M. J., University microfilms, Publication No. 604 (1944); *Microfilm Abstr.*, **5**, No. 2, 9 (1944).
14. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 415 (1943).
15. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

V. PEPTIDASES OF SKIN, LUNG, AND SERUM*

By JOSEPH S. FRUTON

(From the Department of Physiological Chemistry, Yale University, New Haven, and the Laboratories of The Rockefeller Institute for Medical Research, New York)

In previous papers of this series (1–3), it was shown that extracts of animal tissues (swine kidney, beef spleen, etc.) contain a variety of proteolytic enzymes which may be characterized by their specific action on synthetic peptides and peptide derivatives. Several of these tissue enzymes were found to require in their substrates structural elements similar to those present in specific substrates for crystalline pancreatic trypsin. The members of this group of enzymes have been termed trypsinases. Similarly, tissue enzymes related in specificity to pepsin have been named pepsinases and those related to pancreatic carboxypeptidase have been named carboxypeptidases. Enzymes belonging to the same specificity group (*e.g.* the various trypsinases, including pancreatic trypsin) have been designated homospecific enzymes. Thus far, the following groups of homospecific proteolytic enzymes have been identified: Trypsinases (typical substrate, benzoyl-*l*-argininamide); pepsinases (typical substrate, carbobenzoxy-*l*-glutamyl-*l*-tyrosine); carboxypeptidases (typical substrate, carbobenzoxyglycyl-*l*-phenylalanine); and leucine aminopeptidases (typical substrate, *l*-leucinamide).

The trypsinases and pepsinases are endopeptidases; they are capable of hydrolyzing peptide linkages that are not adjacent to a free terminal amino or carboxyl group. Consequently these enzymes are able to hydrolyze central peptide linkages in peptides of high molecular weight as well as in proteins. The carboxy- and aminopeptidases are exopeptidases; they are limited in their action to peptide bonds adjacent to a free α-carboxyl or α-amino group in the substrate.

In view of the limited number of tissue proteolytic enzymes whose specificity has been investigated thus far, it seems desirable to continue the examination of the proteolytic enzymes present in various animal

tissues. In the present communication, data are presented concerning some of the proteolytic enzymes found in extracts of skin and lung and in serum.

Proteolytic Enzymes in Skin Extracts

The recent investigations of Beloff and Peters (4) have provided evidence for the presence, in extracts of rat skin, of at least two proteolytic enzymes. One of these is a proteinase which these authors believe to be of a type not previously described. In addition, they showed that skin extracts are able to hydrolyze *l*-leucylglycylglycine (*l*-LGG), and this action was attributed to peptidases different from the protein-splitting enzyme mentioned before. The fact that extracts of dog skin contain peptidases active toward *l*-LGG has been shown by Zamecnik *et al.* (5).

The experiments described in this section of the present communication deal with the peptidases of rabbit skin. When this tissue is extracted with 2 per cent sodium chloride solution and the extract is clarified with Filter-Cel, the resulting solution exhibits considerable proteolytic activity at pH 7.7 to 8.0 toward substrates such as *l*-LGG, glycylglycylglycine (GGG), and glycyl-*l*-proline (GPro), as may be noted from the data in Table I. The dipeptides *l*-leucylglycine (LG), glycylglycine (GG), and glycyl-*l*-leucine (GL) are split very slowly or not at all, while the typical substrate for leucine aminopeptidase, *l*-leucinamide (LA), is not split appreciably within 24 hours under the experimental conditions employed in this study. It will be seen in Table I that the presence of 0.001 M MnSO$_4$ causes a marked rise in the rate of hydrolysis of LA, thus indicating the presence, in the saline extract, of a manganese-activatable leucine aminopeptidase. It has been shown previously (2, 6) that enzymes belonging to this specificity group hydrolyze not only LA but also LG and *l*-LGG. It may be inferred, therefore, that the increase in the rate of hydrolysis of LG and *l*-LGG by the saline skin extract upon addition of MnSO$_4$ is due, at least in part, to the action of a leucine aminopeptidase. The typical substrate for trypsinases, benzoyl-*l*-argininamide (BAA), and the typical substrate for pepsinases, carbobenzoxy-*l*-glutamyl-*l*-tyrosine (CGluT), are not split measurably in 24 hours, and similar negative results were obtained with carbobenzoxyglycyl-*l*-phenylalanine (CGPha), the typical substrate for carboxypeptidases, as well as with the acylated peptides carbobenzoxy-*l*-leucylglycylglycine (C-*l*-LGG) and carbobenzoxyglycylglycine (CGG).

It would appear, therefore, that of the hitherto recognized specificity types of proteolytic enzymes the only one which can be identified in the saline skin extract is a leucine aminopeptidase. Trypsinase, pepsinase, and carboxypeptidase activity could not be demonstrated, even in the presence of added cysteine, which is known to activate several of the

tissue enzymes belonging to these groups. Beloff and Peters (4) tested
the activity of a saline extract of rat skin toward BAA and carbobenzoxy-*l*-

TABLE I

Proteolytic Activity of Saline Extracts of Rabbit Skin

Enzyme concentration, 0.092 mg. of protein nitrogen per cc. of test solution.

Substrate	pH	Time	Hydrolysis		
			No activator added	0.001 M MnSO₄ present	0.01 M cysteine present
		hrs.	*per cent*	*per cent*	*per cent*
l-Leucylglycylglycine	7.9	1	13	20	12
		2	24	38	27
		3	38	55	37
		5	68	90	
		20	112	126	
	5.0	24	2		1
Glycylglycylglycine	8.0	2.5	29	27	
		5.5	71	65	
		28	123	118	
Glycyl-*l*-proline	8.0	6	6	10	
		24	46	84	
l-Leucylglycine	8.0	5	3	41	3
		20	19	94	18
l-Leucinamide	7.9	3	1	12	3
		24	5	63	3
Glycylglycine	8.2	24	14	17	
Glycyl-*l*-leucine	8.0	24	20	25	
Carbobenzoxy-*l*-leucyl-glycylglycine	7.7	48	0		
Carbobenzoxyglycylglycine	7.9	24	1		
Benzoyl-*l*-argininamide	4.9	24	0		0
	7.5	24	1		
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	5.6	24	2		1
	7.6	24	0		
Carbobenzoxyglycyl-*l*-phenylalanine	5.0	24	1		1
	7.6	24	2		
Benzoylglycinamide	5.0	24			2
	7.5	24	1		
Carbobenzoxy-*l*-serinamide	5.0	24			3
	7.6	24	2		

tyrosylglycinamide, a substrate for pancreatic chymotrypsin, with negative
results. The data presented in Table I support the conclusion of Beloff
and Peters that the endopeptidase activity of skin extracts is of a speci-
ficity type not identified thus far.

Hydrolysis of l-LGG by Skin Extracts—It will be seen from the data in

Table I that the extent of hydrolysis of l-LGG by the saline enzyme extract exceeds that expected for the hydrolysis of one peptide linkage. As noted earlier, the presence of 0.001 M $MnSO_4$ increases the rate slightly. However, when the saline extract is dialyzed against distilled water for 3 days at 4°, and the resulting precipitate is removed, the behavior of the solution toward l-LGG is altered. The data in Table II show that the extent of hydrolysis does not exceed that expected for the hydrolysis of one peptide bond and, furthermore, that the addition of $MnSO_4$ is without noticeable effect on the rate of hydrolysis.

TABLE II

Proteolytic Activity of Water-Dialyzed Extract of Rabbit Skin

Enzyme concentration, 0.066 mg. of protein nitrogen per cc. of test solution.

Substrate	pH	Time	Hydrolysis	
			No MnSO₄ added	0.001 M MnSO₄ present
		hrs.	*per cent*	*per cent*
l-Leucylglycylglycine	7.8	2	48	47
		6	86	88
		24	99	101
		48	102	100
Glycylglycylglycine	8.0	3	53	46
		21	101	100
		46	100	101
Glycyl-l-proline	8.0	3	3	11
		21	16	65
l-Leucylglycine	8.0	20	1	2
l-Leucinamide	7.7	20	2	1
Carbobenzoxy-l-leucyl-glycylglycine	7.6	48	0	
d-Leucylglycylglycine	7.8	48	1	

The hydrolysis of l-LGG by the water-dialyzed extract follows the kinetics of a zero order reaction. As will be seen from the data in Table III, this applies to hydrolysis up to 75 per cent. The kinetics of a given hydrolysis may therefore be defined by a constant K_{LGG}^0 which equals per cent hydrolysis per minute. In Table III, the proteolytic coefficient C is defined as $K/($enzyme concentration (expressed as mg. of protein nitrogen per cc. of test solution)). The data in Table III give a value for C_{LGG}^0 of 5.9 to 6.0, thus showing that the rate constant is proportional to the enzyme concentration in this experiment.

The hydrolysis of only one peptide linkage of l-LGG by the water-dialyzed extract has permitted the determination of the point of enzymatic

cleavage. This was found to be at the peptide bond involving the carbonyl group of the leucyl residue, as indicated by the isolation, from the enzymatic hydrolysate of leucine as a 2-bromotoluene-5-sulfonate and of glycylglycine as a salt of 5-nitronaphthalene-1-sulfonic acid.

The results just presented justify the conclusion that the hydrolysis of l-LGG by the water-dialyzed skin extract is due to the action of a single proteolytic enzyme which may be tentatively named "dermopeptidase."

TABLE III

Kinetics of Hydrolysis of l-Leucylglycylglycine by Dermopeptidase

The pH was maintained at 7.7 in all cases.

Enzyme concentration, protein N per cc. test solution	Time	Hydrolysis	K^0_{LGG}	C^0_{LGG} (average)*
mg.	*min.*	*per cent*		
0.050	60	18	0.30	6.0
	120	36	0.30	
	180	53	0.29	
	240	73	0.30	
0.0375	60	14	0.23	5.9
	120	26	0.22	
	180	40	0.22	
	240	53	0.22	
0.025	60	9	0.15	5.9
	120	18	0.15	
	180	25	0.14	
	240	37	0.15	
0.0165	60	5	0.09	6.0
	120	13	0.11	
	180	18	0.10	
	240	25 .	0.10	
0.010	180	9	0.05	6.0
	300	21	0.07	

* $C^0_{LGG} = K^0_{LGG}$ per mg. protein N.

Since this enzyme does not split LA even in the presence of $MnSO_4$ (*cf.* Table II), it cannot be homospecific with the known leucine aminopeptidases. Dermopeptidase resembles closely an enzyme found by Smith and Bergmann (6) in lead acetate-treated extracts of swine intestinal mucosa. Such extracts are able to hydrolyze l-LGG rapidly and the addition of $MnSO_4$ has no activating effect on this splitting. These authors suggested that the enzyme acting on l-LGG belongs to the group of imidoendopeptidases which would require, in the backbone of their substrates, the groups italicized in the following formula of LGG.

(I) $C_4H_9 \cdot CH \cdot CO - \!\!\!-NH \cdot CH_2 \cdot CO - \!\!\!-NH \cdot CH_2 \cdot COOH$

$\quad\quad\quad\quad\quad | $
$\quad\quad\quad\quad NH_2$
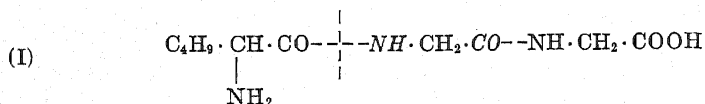
A definite decision as to the specificity requirements of the peptidase in intestinal mucosa has not been reached. However, in view of the finding of similar enzymes in skin and, as will be shown later in this paper, in lung and serum, further data on the specificity of this group of enzymes are most desirable.

In the case of dermopeptidase, it would appear that a terminal free amino group is required in the substrate in order for hydrolysis to occur. This is indicated by the failure of the skin extracts to hydrolyze C-*l*-LGG (*cf.* Tables I and II). It has not been reported whether lead acetate-treated extracts of intestinal mucosa can hydrolyze C-*l*-LGG, and a decision on the essential nature of the terminal amino group therefore cannot be made in this case. As pointed out by Smith and Bergmann (6), an enzyme of the specificity type characterized by Formula I would be expected to hydrolyze other tripeptides containing a central glycine residue. As will be seen from Table II, the water-dialyzed skin extract rapidly hydrolyzes GGG and the hydrolysis stops after one peptide linkage has been split. The presence of 0.001 M MnSO$_4$ results in a slight inhibition of the rate of splitting. It will be of interest to determine in future experiments whether the hydrolysis of GGG is performed by the same enzyme which acts on LGG, namely dermopeptidase.

The antipodal specificity of dermopeptidase is shown by the failure of the water-dialyzed skin extract to hydrolyze *d*-LGG.

Although the specificity type of dermopeptidase and of the related enzyme from intestinal mucosa at present cannot be defined with certainty, it is clear that these enzymes represent a group of proteolytic enzymes different in specificity from the four groups of homospecific enzymes mentioned in the introduction to this paper.

Properties of Dermopeptidase—As will be noted from the data in Table IV, this enzyme is extremely sensitive to slight acidity. Thus, while dermopeptidase is quite stable at 40° for 1 hour at pH values more alkaline than 5, extensive inactivation occurs at more acid pH values. This result raises the possibility that the acid inactivation involves the dissociation of a metal-enzyme complex. While this possibility cannot be excluded, it is of interest that 0.01 M cysteine does not inhibit the action of the enzyme and 0.02 M KCN produces only a slight decrease in activity. No effect on dermopeptidase activity was observed when 0.001 M iodoacetate was present in the test solution.

Hydrolysis of Dipeptides and Leucinamide—As noted above, LG, GG, GL, and LA are hydrolyzed extremely slowly or not at all by the saline

extract of rabbit skin. However, the presence of 0.001 M MnSO$_4$ results in a marked acceleration of enzymatic action on LG and LA and a slight increase in the rate of hydrolysis of GG and GL. The strong manganese activation of the hydrolysis of LG and LA indicates the presence, in the saline extract, of a leucine aminopeptidase with properties similar to that found in swine intestinal mucosa (6). The slight increase in the rate of hydrolysis of l-LGG by the saline extract when MnSO$_4$ is added indicates that leucine aminopeptidase participates, to some extent, in the hydrolysis of this substrate. Thus, in the crude extract, at least two enzymes are involved in the hydrolysis of l-LGG, a manganese-activatable aminopeptidase as well as dermopeptidase. In addition, the GG liberated in the

TABLE IV

pH Stability of Dermopeptidase

The pH of the enzyme solution was adjusted by the addition of suitable amounts of 0.1 N hydrochloric acid to the veronal buffer-enzyme mixture. After 1 hour at 40°, the pH was readjusted to pH 7.6 and the substrate (l-leucylglycylglycine) was added. Enzyme concentration, 0.033 mg. of protein nitrogen per cc. of test solution.

pH of enzyme solution	K^0_{LGG}
7.5	0.19
7.2	0.19
6.7	0.19
6.3	0.20
5.9	0.19
5.3	0.18
4.5	0.06
4.2	0.015
3.5	0.00
3.0	0.00

course of this combined enzyme action is probably hydrolyzed to some extent, although this action does not appear to be subject to activation by manganese. The difference in the behavior of leucyl peptides and glycyl peptides toward added MnSO$_4$ indicates the presence, in the saline extract, of peptidases other than leucine aminopeptidase and dermopeptidase.

It was mentioned before that dialysis of the saline skin extract against distilled water destroys the enzymatic activity toward the dipeptides and LA even when the test is performed in the presence of MnSO$_4$. Attempts to extract, with 2 per cent saline, the manganese-activatable enzymes from the precipitate formed during dialysis were unsuccessful. Apparently these enzymes undergo irreversible inactivation on dialysis.

Hydrolysis of Glycyl-l-proline—It has been shown previously (7) that extracts of intestinal mucosa contain an enzyme specifically adapted to the

hydrolysis of peptide linkages involving the imino nitrogen of l-proline. This enzyme, named prolidase, has been found by Smith and Bergmann (6) to hydrolyze not only glycyl-l-proline but glycyl-l-hydroxyproline as well. They showed further that the prolidase of swine intestinal mucosa is acti-

TABLE V

Proteolytic Activity of Extracts of Human Skin

Substrate	pH	Time	Hydrolysis			
			Saline extract, 0.042 mg. protein N per cc. test solution		Water-dialyzed extract, 0.009 mg. protein N per cc. test solution	
			No MnSO₄ added	0.001 M MnSO₄ present	No MnSO₄ added	0.001 M MnSO₄ present
		hrs.	*per cent*	*per cent*	*per cent*	*per cent*
l-Leucylglycylglycine	7.9	2	44	68	12	14
		4	85	109	25	26
Glycylglycylglycine	8.0	2.5	45	45	11	10
		4.5	73	72	19	16
		8			36	28
Glycyl-l-proline	8.0	2.5		9		
		6.5		16		
		23	12	63	6	36
l-Leucylglycine	8.0	2.5	32	45		
		4.5	54	65		
		23			0	0
l-Leucinamide	7.9	2	0	17		
		4	2	38		
		23				1
Glycylglycine	8.0	4.5	10	26		
		23	22	40	0	1
Glycyl-l-leucine	7.9	4.5	55	43	2	2
Benzoyl-l-argininamide	4.9	24	0*			
	7.5	24	1			
Carbobenzoxy-l-iso-glutamine	5.2	24	1*			
	7.4	24	2			
Benzoylglycinamide	7.5	24	0			

* 0.01 M cysteine present.

vated by manganese ions. It was of interest to find, in the present study, that saline skin extracts contain appreciable prolidase activity, which is also increased on addition of MnSO₄. As will be noted from Table II, the prolidase of skin is not inactivated appreciably on dialysis against distilled water, and thus accompanies the dermopeptidase in the water-dialyzed extract.

Proteolytic Activity of Extracts of Human Skin—Saline extracts of human skin, obtained at autopsy, showed an enzymatic behavior essentially similar to that previously found with extracts of rabbit skin (cf. Table V).

Here again, the presence of a special enzyme capable of hydrolyzing *l*-LGG could be demonstrated. As will be seen from Table V, this enzyme does not require the addition of manganese ions for activity and is stable to dialysis against distilled water. The presence of a manganese-activatable leucine aminopeptidase and of prolidase could be demonstrated in the saline extract. As in the case of the rabbit skin extract, dialysis against distilled water destroyed the leucine aminopeptidase activity.

TABLE VI

Proteolytic Activity of Saline Extracts of Rabbit Lung

Enzyme concentration, 0.15 mg. of protein nitrogen per cc. of test solution.

Substrate	pH	Time	Hydrolysis		
			No activator added	0.001 M MnSO₄ present	0.01 M cysteine present
		hrs.	*per cent*	*per cent*	*per cent*
l-Leucylglycylglycine	7.8	1	29	44	30
		2	57	77	54
Glycylglycylglycine	7.8	2	19	15	
		4	40	33	
l-Leucylglycine	7.9	3	10	54	
l-Leucinamide	7.8	2	4	28	
		4	7	51	
Glycylglycine	7.7	4.5	10	12	
Glycyl-*l*-leucine	7.8	4.5	21	25	
Glycyl-*l*-phenylalanine	7.8	4.5	18	21	
Benzoyl-*l*-argininamide	5.0	20			4
	7.5	20	2		
Carbobenzoxy-*l*-iso-glutamine	5.4	20			0
Carbobenzoxy-*l*-gluta-myl-*l*-tyrosine	5.2	20	0		
Carbobenzoxyglycyl-*l*-phenylalanine	5.3	20			0
	7.6	20	0		0

Proteolytic Enzymes in Lung Extracts

The data in Table VI show several points of similarity in the proteolytic activity of extracts of rabbit lung as compared with that of skin extracts. Thus *l*-LGG and GGG are split rapidly in the absence of added manganese ions, and addition of MnSO₄ leads to an accelerated hydrolysis in the case of *l*-LGG but not of GGG. The activation of *l*-LGG splitting by manganese may be attributed to the participation of a leucine aminopeptidase whose presence in the lung extract is shown by the rapid hydrolysis of LA and LG in the presence of MnSO₄. As in the case of the skin extracts, no evidence could be found for the presence, in lung extracts, of proteolytic

enzymes homospecific with the known trypsinases, pepsinases, or car-
boxypeptidases.

Dialysis of a saline extract of rabbit lung against distilled water for 4
days at 4° leads to the loss of leucine aminopeptidase activity but the
ability to hydrolyze *l*-LGG is retained. The addition of 0.001 M MnSO4
does not increase the rate of hydrolysis. It may be concluded, therefore,
that rabbit lung extracts contain a proteolytic enzyme related to the
dermopeptidases of rabbit and human skin.

Proteolytic Enzymes of Serum

Numerous workers have studied the hydrolysis of peptides by sera of
various animals. Grassmann and Heyde, in particular, showed that sera of
rabbits, swine, horses, and humans caused rapid hydrolysis of LGG and
a slow splitting of LG (8). The optimum pH for the hydrolysis of LGG
was found to be about 7.5. On standing, the sera lost their activity toward
the dipeptide but retained the ability to split the tripeptide. For this
reason, the authors concluded that there were present, in serum, at least
two enzymes, one of which was classified as a dipeptidase and the other as
an aminopolypeptidase. Abderhalden and Hanson (9) found no car-
boxypeptidase activity in rabbit plasma or serum when chloroacetyl-*l*-
tyrosine and several N-acylated peptides were used as substrates. Masch-
mann (10), in an extensive study of the hydrolysis of di- and tripeptides
by sera of sheep, rabbits, and guinea pigs, found that the addition of man-
ganese, magnesium, or cobalt ions markedly increased the rate of splitting,
especially in the case of the dipeptides. Considerable variability in the
response to these metals was observed, depending on the nature of the
metal, the structure of the substrate, and the type of serum which was
employed.

Hydrolysis of l-LGG—In the present communication, additional data are
presented concerning the serum enzymes which hydrolyze *l*-LGG. The
findings suggest a relationship between these enzymes and those found in
extracts of skin and lung.

It will be noted from Table VII that rabbit serum hydrolyzes *l*-LGG
rapidly at pH 7.6 to 7.7 and that the observed rate of splitting follows the
kinetics of a zero order reaction. The rate may therefore be expressed by
the constant K^0_{LGG} which is defined as per cent hydrolysis per minute.
Within the limits of enzyme concentration given in Table VII, the value of
the constant is proportional to the amount of serum per cc. of the test
solution.

A surprisingly constant value for K^0_{LGG} was noted when fifteen normal
rabbits of varying size (2400 to 3750 gm.) were tested for their serum
peptidase level. This value was found to be 0.18 ± 0.01 for 0.1 cc. of rabbit

serum per cc. of test solution. In the case of several normal rabbits, successive blood samples were drawn at intervals of 24, 48, and 72 hours. The peptidase level was the same (0.18 ± 0.01) for all the blood samples. It may be mentioned in this connection that Grassmann and Heyde (8) observed a similar constancy of the serum peptidase level in normal human subjects when LGG was used as the substrate. From the data of Grassmann and Heyde, an approximate value for the peptidase index of normal human serum may be calculated. Assuming zero order kinetics and proportionality between rate and enzyme concentration, K_{LGG}^0 is found to

TABLE VII

Hydrolysis of l-Leucylglycylglycine by Rabbit Serum

Rabbit serum per cc. test solution	Added substance	Time	Hydrolysis	K_{LGG}^0 (average)
cc.		*min.*	*per cent*	
0.2	None	40	14	0.36
		80	30	
		120	43	
0.1	"	70	12	0.18
		170	32	
		300	65	
0.05	"	70	7	0.10
		170	15	
		300	34	
0.2	0.001 M $MnSO_4$	60	24	0.39
		120	46	
0.2	0.01 " cysteine	80	25	0.32
		120	39	
0.2	0.02 " cyanide	80	18	0.24
		120	31	
0.2	0.001 " iodoace-tate	80	31	0.38
		120	44	

be 0.035 for 0.1 cc. of human serum per cc. of test solution. This value is one-fifth of that found for rabbit serum.

The presence of 0.001 M $MnSO_4$ did not increase appreciably the rate of hydrolysis of l-LGG by rabbit serum. This result agrees with the finding of Maschmann (10) for rabbit serum. It should be added that, with guinea pig serum, he found activation of LGG hydrolysis by $MnSO_4$. This latter observation has been confirmed in the course of the present study. In agreement with Maschmann's data, appreciable inhibition of LGG hydrolysis by rabbit serum was observed when 0.02 M cyanide was present. However, the addition of 0.01 M cysteine caused only slight inhibition and the addition of 0.001 M iodoacetate did not influence appreciably the enzymatic activity.

It was noted earlier in this paper that the dermopeptidase of rabbit skin is extremely sensitive to slight acidity, and a similar result has been observed for the rabbit serum peptidase. Thus, exposure of rabbit serum to pH 3.75 at 40° for 1 hour led to complete loss of enzyme activity. Dialysis against distilled water for 6 days at 4° did not destroy the ability of rabbit serum to split l-LGG.

For comparative purposes, experiments were also performed on the hydrolysis of l-LGG by horse serum (cf. Table VIII). In this case, the enzymatic activity per cc. of serum was somewhat less than that of rabbit serum. Furthermore, although no appreciable activation by 0.001 M MnSO$_4$ was noted, there was considerable inhibition on addition of cysteine. It was of interest to note that, when MnSO$_4$ and cysteine were added together, the activity toward l-LGG was almost completely inhibited.

TABLE VIII

Hydrolysis of l-Leucylglycylglycine by Horse Serum

0.2 cc. of serum was present per cc. of test solution; pH, 7.8.

Added substance	Time	Hydrolysis	K^0_{LGG}
	min.	*per cent*	
None	180	22	0.12
	330	39	0.12
0.001 M MnSO$_4$	180	23	0.13
	330	40	0.12
0.01 " cysteine	180	13	0.07
	325	20	0.07
0.001 " MnSO$_4$ +	180	2	0.01
0.01 " cysteine	300	4	0.01

Similar inhibition of l-LGG hydrolysis by MnSO$_4$ plus cysteine was also observed in the case of rabbit serum and rabbit lung, although in these cases the magnitude of the effect was not as great as that found for horse serum. No explanation for these effects can be offered at present.

Specificity of Serum Peptidases—As noted earlier, Grassmann and Heyde (8) attributed the hydrolysis of LGG to a serum aminopolypeptidase. This was in keeping with the classification of the proteolytic enzymes current in 1930, when these enzymes were defined on the basis of the chain length of the substrates which they attacked. It has since become clear that this classification is inadequate and must be replaced by one in which the specificity is defined in terms of the backbone and side chain requirements in the substrate (11). In the section of this paper dealing with the proteolytic enzymes of the skin, evidence was presented to show that the action of skin extracts on l-LGG is due to at least two enzymes, a man-

ganese-activatable leucine aminopeptidase and another enzyme, of different specificity, named dermopeptidase. The leucine aminopeptidase activity of rabbit serum is low, as is evidenced by the slow hydrolysis of LA and LG even in the presence of added manganese ions. These results, coupled with the data of Grassmann and Heyde and of Maschmann, give strong support for the participation of at least two enzymes in the hydrolysis of *l*-LGG by rabbit serum, and presumably other sera as well. One of these may be classified as a manganese-activatable aminopeptidase, while the other, apparently present in a more active state, is related in specificity and other properties to the dermopeptidases. The relative amounts of these two enzymes would determine the response of a given serum to the addition of metal activators and may explain the variability observed by Maschmann in the activation behavior of sera of different species.

"Proteinases" and "Antiproteinases" of Serum—In 1903, Delezenne and Pozerski (12) showed that the proteolytic activity of serum could be increased by treatment with chloroform. More recently, Milstone (13) and Christensen (14) have demonstrated that serum contains an inactive proteinase which may be activated by streptococcal "fibrinolysin." Christensen and MacLeod (15) have shown further that the enzyme activity elicited by chloroform or by fibrinolysin is due to the same proteinase, and the name "plasmin" has been suggested for this enzyme. Apparently plasmin has a substrate specificity different from that of pancreatic trypsin. The last named workers have shown also that the activation of plasmin by streptococcal fibrinolysin is a catalytic reaction analogous to the activation of trypsinogen by enterokinase. For this reason, the fibrinolysin is termed "streptokinase." On the other hand, the activation of plasmin by chloroform treatment is attributed to the removal of an inhibitor present in serum followed by autocatalytic activation of plasmin. Taylor *et al.* (16) have provided experimental data on the distribution of plasmin in fractions of human plasma. Ferguson (17) and Tagnon *et al.* (18) have discussed the possible rôle of this enzyme in blood coagulation.

The current interest in the physiological rôle of the plasma proteinases suggested the desirability of finding substrates of known structure for these enzymes. Accordingly, rabbit serum was allowed to act on a variety of peptide derivatives which had previously been found to be hydrolyzed by proteinases (endopeptidases). These substances included BAA, hippuryl-*l*-argininamide (HAA), CGluT, carbobenzoxy-*l*-isoglutamine (CiG), carbobenzoxy-*l*-serinamide (CSA), and benzoylglycinamide (BGA). Of this group, only BGA was found to be split appreciably by rabbit serum (*cf.* Table IX). Although hydrolysis of BGA was noted at pH 5.3 to 5.4, both in the presence and the absence of cysteine, the substrate was

hydrolyzed more rapidly at pH 7.6. The hydrolysis of BGA by rabbit serum occurs at the amide linkage involving the carbonyl of the glycine residue, as was shown by the isolation of hippuric acid (m.p. 186–187°) from the hydrolysate and the finding of the quantity of ammonia expected on the basis of the extent of liberation of carboxyl groups.

As has been shown previously (19), BGA is hydrolyzed rapidly by cysteine-activated papain to yield hippuric acid and ammonia. It would appear, therefore, that serum contains an endopeptidase which is homospecific with an enzymatic component of papain. Since these enzymes

TABLE IX

Action of Rabbit Serum on Substrates for Endopeptidases

0.2 cc. of serum was present per cc. of test solution.

Substrate	pH	Time	Hydrolysis
		hrs.	*per cent*
Benzoylglycinamide	5.4	18	20*
		42	30*
	5.3	18	15
		42	33
	7.6†	18	27
		42	47
Benzoyl-l-argininamide	5.0	20	2*
	7.7†	20	1
Hippuryl-l-argininamide	7.8†	20	0
Carbobenzoxy-l-glutamyl-l-tyrosine	5.6	20	1
	7.8†	20	0
Carbobenzoxy-l-isoglutamine	5.5	20	0*
	7.6†	20	1
Carbobenzoxy-l-serinamide	5.4	20	2*
	7.6†	20	4

* 0.01 M cysteine present.

† 0.02 M phosphate used as buffer.

do not fit into any of the specificity groups of proteolytic enzymes identified thus far, it may be concluded that the hydrolysis of BGA involves the action of a type of endopeptidase different from those designated as trypsinases or pepsinases.

In view of the possibility that the proteolytic activity of rabbit serum toward one or more of the peptide derivatives may be masked either through inhibition or by inadequate activation, samples of rabbit serum were shaken with chloroform according to the directions of Tagnon *et al.* However, no appreciable proteolytic activity toward BAA, CGluT, CiG, or CSA was observed following such treatment, and the activity toward BGA was not measurably increased.

Through the kindness of Dr. Edwin J. Cohn, an opportunity was provided to test the proteolytic activity of fractions of human plasma toward several peptide derivatives. Preliminary experiments have shown that, in addition to activity toward LGG, the globulin fractions III and IV contained enzymatic activity toward BGA. This work is being continued.

Work on these proteinases has been made difficult by the complex composition of plasma and especially by the presence of inhibitors for proteolytic enzymes. The ability of small amounts of serum to inhibit the action of pancreatic trypsin has long been recognized, although the nature of substances responsible for this effect is still under discussion (20). Recently, Beloff (21) has shown that a component of the albumin fraction of plasma acts as a powerful inhibitor of the skin proteinase described by Beloff and Peters (4).

DISCUSSION

The data in the present communication indicate the presence, in extracts of skin and lung tissue and in serum, of peptidases capable of acting on LGG but different from the hitherto recognized leucine aminopeptidases. There are, in addition, reports in the literature which suggest the presence of similar enzymes in intestinal mucosa (6), in lymph and muscle (5), and in leucocytes and lymphocytes (22, 23). The widespread distribution of these enzymes in tissues raises the possibility that they may have a common origin and that they are obtained on extraction of a particular tissue such as skin or lung, not from the characteristic cells of that tissue but from cells such as leucocytes or lymphocytes present in the tissue. Furthermore, the presence of these peptidases in serum suggests that they are liberated into the circulating body fluids in the course of the disintegration of cells such as lymphocytes. This view is supported by the finding[1] that the injection of mice or rabbits with pituitary adrenotropic hormone or with adrenal cortical extracts leads to a marked increase in the capacity of the sera of these animals to split LGG. The rise in serum peptidase activity is seen during the period following hormone administration when the rate of release of protein from lymphoid tissue to the circulation is augmented (24).

The author wishes to express his thanks to Miss Nancy North and Miss Rosalind Joseph for valuable assistance in this investigation.

EXPERIMENTAL

Skin Peptidases—30 gm. of skin were removed from the back of an exsanguinated rabbit and the adhering connective tissue was scraped off as

[1] Holman, H., White, A., and Fruton, J. S., unpublished experiments.

completely as possible. The skin tissue was cut into small pieces, 100 cc. of 2 per cent sodium chloride were added, and the suspension was homogenized in a Waring blendor. An additional 100 cc. of 2 per cent sodium chloride were added and the mixture was stirred for 3 hours at room temperature. The suspension was filtered through Schleicher and Schüll No. 1450½ filter paper and clarified by means of Filter-Cel. The resulting clear solution was used in the experiments reported in Table I. In order to remove leucine aminopeptidase activity, the saline extract was dialyzed against distilled water for 3 days at 4°. The insoluble material which settled out was removed by filtration and the clear filtrate was used for the experiments reported in Tables II to IV. The water-dialyzed enzyme solutions were kept in the refrigerator with a small amount of toluene, and showed no significant decrease in activity over a period of 3 weeks.

Extracts of human skin obtained on autopsy were prepared in the same manner as described above for rabbit skin.

Lung Peptidases—The lungs (17.5 gm.) of two exsanguinated rabbits were removed, washed with saline, and minced in a Waring blendor. The minced tissue was stirred at room temperature with 200 cc. of 2 per cent sodium chloride for 3 hours, and the mixture was left overnight in the refrigerator. The suspension was then filtered, and the resulting clear filtrate was used for enzyme experiments.

Measurement of Enzyme Activity—In all cases, the substrate concentration was 0.05 mM per cc. of test solution. Unless otherwise stated, the pH was maintained by means of 0.02 M citrate buffer at pH 4.9 to 5.6, and with 0.02 M veronal buffer at pH 7.5 to 8.2. The flasks containing substrate, buffer, and enzyme were kept in a water thermostat at 39°. The rate of hydrolysis was followed by measurement of the liberation of carboxyl groups by the method of Grassmann and Heyde (25). The data in Tables I to IX are given in terms of per cent of the hydrolysis expected on the complete splitting of one peptide linkage.

Isolation of Leucine and Glycylglycine following Hydrolysis of l-LGG by Dermopeptidase—10 cc. of a solution containing 306 mg. of *l*-LGG and 2 cc. of the water-dialyzed skin extract (0.21 mg. of protein nitrogen) were incubated for 24 hours at 39°. The solution was then acidified to pH 2 with dilute hydrochloric acid and evaporated to dryness under reduced pressure. The residue was dissolved in 5 cc. of hot water and 400 mg. of sodium 2-bromotoluene-5-sulfonate (26) were added. After standing overnight in the refrigerator, the leucine bromotoluene sulfonate which had separated was filtered and washed with cold water. Yield, 430 mg. The salt was recrystallized from hot water for analysis.

$C_{13}H_{20}NO_5SBr.$　　Calculated.　C 40.8, H 5.3, N 3.7
382.3　　　　　　　　Found.　　" 40.7, " 5.2, " 3.8

The addition of 540 mg. of 5-nitronaphthalene-1-sulfonic acid (26) to the mother liquor and washings from the isolation of leucine yielded the characteristic crystals of glycylglycine nitronaphthalene sulfonate. Yield, 352 mg. The salt was recrystallized from hot water for analysis.

$C_{14}H_{15}N_3O_8S$. Calculated. C 43.6, H 3.9, N 10.9
385.3   Found.   " 43.6, " 3.8, " 11.1

SUMMARY

1. Saline extracts of rabbit skin contain several proteolytic enzymes. One of these hydrolyzes l-leucylglycylglycine at pH 7.8 and does not require activation by manganese ions or cysteine. This enzyme, named dermopeptidase, has a specificity different from that of leucine aminopeptidase, which is also present in saline extracts of rabbit skin. On dialysis of the saline extract against distilled water dermopeptidase activity is retained but leucine aminopeptidase activity is lost. Prolidase is present in skin extracts, but no evidence could be obtained for the presence of a carboxypeptidase or of proteinases of known specificity type.

2. Extracts of rabbit lung contain an enzyme which hydrolyzes l-leucylglycylglycine and which is related to dermopeptidase.

3. The sera of several animals contain an enzyme which splits l-leucylglycylglycine and which bears many points of similarity to dermopeptidase. It was found that rabbit serum hydrolyzes benzoylglycinamide through the action of a hitherto unidentified proteolytic enzyme.

4. Attention is drawn to the widespread distribution of enzymes which act on l-leucylglycylglycine and which are apparently related in specificity and other properties to dermopeptidase.

BIBLIOGRAPHY

1. Fruton, J. S., and Bergmann, M., J. Biol. Chem., **130**, 19 (1939).
2. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., J. Biol. Chem., **138**, 249 (1941).
3. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., J. Biol. Chem., **141**, 763 (1941).
4. Beloff, A., and Peters, R. A., J. Physiol., **103**, 461 (1945).
5. Zamecnik, P. C., Stephenson, M. L., and Cope, O., J. Biol. Chem., **158**, 135 (1945).
6. Smith, E. L., and Bergmann, M., J. Biol. Chem., **153**, 627 (1944).
7. Bergmann, M., and Fruton, J. S., J. Biol. Chem., **117**, 189 (1937).
8. Grassmann, W., and Heyde, W., Z. physiol. Chem., **188**, 69 (1930).
9. Abderhalden, E., and Hanson, H., Fermentforschung, **15**, 382 (1937).
10. Maschmann, E., Biochem. Z., **308**, 359 (1941).
11. Bergmann, M., and Fruton, J. S., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1**, 63 (1941).
12. Delezenne, C., and Pozerski, E., Compt. rend. Soc. biol., **55**, 690 (1903).
13. Milstone, H., J. Immunol., **42**, 109 (1941).

14. Christensen, L. R., *J. Gen. Physiol.*, **28**, 363 (1945).
15. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, **28**, 559 (1945).
16. Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., McDonald, A. H., and Minot, G. R., *J. Clin. Invest.*, **24**, 698 (1945).
17. Ferguson, J. H., *J. Gen. Physiol.*, **24**, 169 (1940).
18. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, **21**, 525 (1942).
19. Bergmann, M., Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, **111**, 225 (1935).
20. Grob, D., *J. Gen. Physiol.*, **26**, 405 (1943).
21. Beloff, A., *Biochem. J.*, **40**, 108 (1946).
22. Husfeldt, E., *Z. physiol. Chem.*, **194**, 137 (1931).
23. Opie, E. L., *Physiol. Rev.*, **2**, 552 (1922).
24. White, A., and Dougherty, T. F., *Endocrinology*, **36**, 207 (1945).
25. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
26. Doherty, D. G., Stein, W. H., and Bergmann, M., *J. Biol. Chem.*, **135**, 487 (1940).

A STUDY WITH ISOTOPIC CARBON OF THE ASSIMILATION OF CARBON DIOXIDE IN THE RAT*

By ADELAIDE M. DELLUVA AND D. WRIGHT WILSON

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

That assimilation of carbon dioxide is an important reaction in heterotropic organisms was pointed out by Wood and Werkman (1) who subsequently clarified the mechanism by means of C^{13} (2). The first suggestion that mammalian tissue can fix carbon dioxide was made by Ruben and Kamen (3). Further evidence for the incorporation of carbon dioxide has been provided by Evans and Slotin (4) who demonstrated the formation of radioactive α-ketoglutarate by pigeon liver mince in a medium containing pyruvate and radioactive bicarbonate, by Wood *et al.* (5) through studies on pyruvate dissimilation in liver slices, by Evans and coworkers (6) in work on enzyme systems obtained from cell-free extracts of pigeon liver, and by Lorber *et al.* (7) in experiments on glycogen formation by the mammalian heart. Experimentation with the intact animal has been more difficult because of the attendant dilution of the labeled carbon. The amount of non-isotopic carbon dioxide resulting from metabolic processes greatly exceeds that of the injected isotopic carbon dioxide. Nevertheless, Solomon and coworkers (8) using radioactive carbon, C^{11}, have succeeded in showing the incorporation of the carbon of carbon dioxide in the glycogen and probably also in the soft tissues of the rat. In experiments conducted on the rat with the use of isotopic sodium bicarbonate labeled with C^{13}, Wood *et al.* (9) have shown uptake of carbon dioxide in glycogen and have determined the location of the labeled carbon in the glucose obtained by the hydrolysis of the glycogen. Proof that the synthesis of urea involves fixation of carbon dioxide has been provided (10, 11).

Although various aspects of the metabolism of the four and five carbon dicarboxylic acids in relation to the carbon dioxide assimilation process have been studied and the location of the isotopic carbon in the molecules determined (12), the presence of the labeled carbon in the corresponding amino acids has never been adequately demonstrated. Evans and Slotin

(4) in experiments on the utilization of carbon dioxide by pigeon liver found that a part of the residual radioactivity in the pigeon liver tissue could be released by the action of ninhydrin and chloramine-T, two reagents which are known to liberate the carboxyl group α to the amino group of amino acids.

An attempt was made in this study to determine the relationship of glutamic and aspartic acids and arginine to the assimilation of carbon dioxide as well as to study incorporation of isotopic carbon in other compounds.

EXPERIMENTAL

In order to devise a practicable method for the administration of isotopic carbon dioxide, a preliminary experiment was performed in which isotopic carbon was fed to two immature rats, 29 days of age, with an average weight of 52 gm. The labeled carbon was in the form of calcium carbonate which was mixed with the diet of the rats at a level of 3 per cent.[1] The diet was fed for a period of 10 days. The total amount of food consumed was 106 gm., containing 3.18 gm. of calcium carbonate, or 31.8 millimoles of carbon which contained 8.6 atom per cent excess C^{13}. During each day, four 2 hour samples of respiratory carbon dioxide were collected as barium carbonate. The urines were pooled and the feces were collected and dried. At the end of the experimental period the rats weighed an average of 69 gm., a gain of 17 gm. for each rat. The animals were killed by exsanguination. The urea of the urine, the carbonate of the bones and the feces, and the proteins of the liver and muscle were analyzed for isotopic carbon.

The following results were obtained (the isotope concentration is expressed as atom per cent excess C^{13}): urea carbon, 0.06; feces carbonate, 6.51; bone carbonate, 0.00 to 0.03; liver glutamic acid, α-carboxyl carbon, 0.03; liver aspartic acid, both carboxyls, 0.02. The same amino acids from the muscle proteins contained no detectable excess of isotope. Since the analytical method had a probable error of ±0.02 atom per cent, the latter figures are without significance.

The concentration of labeled carbon in the materials isolated was so small that the method of introducing the isotopic carbon was changed. Intraperitoneal injection of a solution containing labeled sodium bicarbonate was used.

[1] The diet consisted of 70 per cent dextrin, 10 per cent casein, 13.5 per cent cottonseed oil, 0.5 per cent choline chloride, and 6.0 per cent salt mixture. The salt mixture was 3.0 gm. of calcium carbonate, 1.0 gm. of sodium chloride, 1.64 gm. of monopotassium phosphate, 0.23 gm. of magnesium sulfate, 0.18 gm. of potassium iodide, and 0.110 gm. of ferrous sulfate. Cod liver oil was administered by mouth, and the necessary water-soluble vitamins (thiamine, riboflavin, pantothenic acid, and pyridoxine) were given as a water solution.

Isotopic sodium bicarbonate was prepared from barium carbonate, containing 14.0 atom per cent excess C^{13}. The barium carbonate was obtained from methane in which the isotope had been concentrated by thermal diffusion. The bicarbonate solution was made isotonic by appropriate dilution.

An adult female rat weighing 270 gm. was injected intraperitoneally with 5 ml. portions of isotonic sodium bicarbonate, containing 14.0 atom per cent excess C^{13}, at hourly intervals for 18 hours. Half an hour after the last injection the animal was anesthetized with ether and 3 ml. of blood were collected from a severed carotid artery.

TABLE I

C^{13} Concentration of Respiratory Carbon Dioxide

Sample No.	Hr. of experiment	Atom per cent excess C^{13}
1	2nd	0.22
2	4th	0.32
3	6th	0.44
4	8th	0.70
5	8th	0.60
6	11th	0.31
7	13th	0.32
8	15th	0.42
9	17th	0.70
10	17th	0.52
11	18th	0.80

Samples 4 and 9 were collected beginning 15 minutes after the injection of the bicarbonate. All the other collections were begun 30 minutes after injection, except Sample 11, which was begun 10 minutes after the last injection. Each collection of the respiratory carbon dioxide was made for a 15 minute period.

During the course of the experiment, in which the rat consumed 3.5 gm. of stock diet (Steenbock stock diet, modified (13)), several samples of respiratory carbon dioxide were obtained. For the collection of these samples, the rat was placed in a glass desiccator and the respiratory carbon dioxide was trapped by aspirating the expired air through a sintered glass disk into a column of dilute sodium hydroxide made by mixing carbonate-free alkali with carbon dioxide-free water. (Air was drawn into the system through a trap of soda lime in order that atmospheric carbon dioxide might be removed.) The carbonate so formed was precipitated by an excess of saturated barium chloride and the barium carbonate centrifuged and washed with CO_2-free water until the washings were neutral to litmus. The various samples of carbonate were analyzed for isotope. The results are found in Table I.

There was a steady rise in concentration of labeled carbon in the respiratory carbon dioxide until the 11th hour (Sample 6) of the experiment. At this time there was a drop in isotope concentration of the carbon dioxide which may probably be ascribed to increased activity of the animal.

Carbonate from the pooled feces and the pooled urine was isolated as follows: The feces were collected in acetone and then dried and ground. A 300 mg. sample was acidified with HCl and the evolved carbon dioxide collected as barium carbonate. A 10 ml. aliquot of the pooled urine, the total volume of which was 70 ml., was acidified with acetic acid and the carbon dioxide liberated from the urinary carbonate collected as barium carbonate. The relation of C^{13} to total C was determined (Table II).

The urine used for the recovery of carbonate was combined with the remainder. The total volume was made acid with acetic acid and evaporated to dryness *in vacuo* to remove all carbonate. The residue was

TABLE II

C^{13} *Concentration in Blood, Urine, Feces, and Bone*

	atom per cent excess		atom per cent excess
Blood bicarbonate............	0.64	Bone carbonate	
Urinary bicarbonate..........	0.67	Epiphyses..................	0.08
Urea carbon.................	0.34	Subepiphyses..............	0.10
Feces carbonate.............	0.02	Diaphyses.................	0.08

dissolved in water and made up to a volume of 10 ml. A 2 ml. aliquot was slightly acidified, aerated for 1 hour to remove any possible traces of carbonate, adjusted to pH 6.8 with freshly prepared phosphate buffer, and digested at 50° with urease (a solution of the crude crystalline material prepared according to Sumner and Sisler (14)). The carbon dioxide liberated from the urea was isolated as barium carbonate and the atom per cent excess C^{13} determined (see Table II).

It is of interest to note that the concentration of isotope in the bicarbonate of the urine was about the same as that of the blood. This is probably a coincidence but it emphasizes the thought that the bicarbonate excretion in urine was undoubtedly maximum soon after the injections when there were maximum concentrations of isotope in the circulating blood. The isotope concentration of the carbonate in the feces was small.

After the animal was killed by exsanguination and the blood collected for bicarbonate isolation, the liver was quickly removed and ground in a Waring blendor with 5 per cent trichloroacetic acid in order to precipitate the proteins. The kidney, spleen, and part of the small intestine, combined

as one fraction, and the skeletal muscle, as another fraction, were similarly treated. No glycogen was found in any of these fractions when they were treated with 95 per cent ethanol.

The long bones of the fore and hind legs were boiled in dilute ammonia water in order to remove the remaining muscle and connective tissue. The bones were thoroughly dried, separated into three fractions (epiphyses, subepiphyses, and diaphyses), and the carbonate portions isolated for isotope analysis (see Table II).

. The precipitated proteins were dried and made fat-free by extraction for 8 hours with hot acetone. After removal of the fat, the proteins from the liver, muscle, and kidney fractions were refluxed for 20 hours with 10 times their weight of 20 per cent HCl. Upon completion of hydrolysis the material from each portion was filtered to remove humin and evaporated *in vacuo* to remove excess HCl. The residue was freed of chloride by treatment with silver carbonate and the excess silver removed by means of hydrogen sulfide. The solution was filtered, evaporated to a small volume, adjusted to pH 6, and allowed to remain in the refrigerator for 48 hours to insure complete precipitation of tyrosine.

After removal of the tyrosine, the filtrate was made alkaline (pH 9 to 10) with hot saturated barium hydroxide and the barium salts of the glutamic and aspartic acids precipitated by the addition of 3 volumes of absolute ethanol. From the barium salts, the glutamic and aspartic acids were isolated as the hydrochloride and copper salt respectively. The materials were recrystallized and analyzed.

The nitrogen analyses (Kjeldahl) were as follows: glutamic acid hydrochloride, theory 7.6 per cent; found, from liver protein 7.6 per cent; from muscle protein 7.5 per cent; from the protein of the combined kidney, spleen, and portion of the small intestine (hereafter referred to as the kidney-spleen protein) 7.4 per cent; copper aspartate, theory 7.1 per cent; found, from liver protein 7.1 per cent; from muscle protein 7.1 per cent; from kidney-spleen protein 6.9 per cent.

The glutamic and aspartic acids were decarboxylated by means of ninhydrin according to the method of Van Slyke *et al.* (15) and the CO_2 collected as carbonate. The isotope analyses of the isolated carbonates are given in Table III.

Isotopic carbon was incorporated into amino acids of liver proteins. There was hardly a significant incorporation in the kidney-spleen and muscle proteins. Although the concentrations of C^{13} in the glutamic and aspartic acids of the liver are low, they are significantly above the normal value. It should be remembered that isotopic amino acids formed during metabolism are diluted considerably by non-isotopic amino acids when they are incorporated into the protein molecule.

The filtrates from the barium salts of glutamic and aspartic acids were freed of barium, evaporated to a small volume, adjusted to a pH of 4 with H_2SO_4, and treated with excess flavianic acid. The precipitate of arginine flavianate was collected after 24 hours in the refrigerator. The flavianate was dissolved in hot dilute HCl and the flavianic acid extracted by means of n-butyl alcohol. Subsequent evaporation to dryness *in vacuo* removed excess HCl. The amidine carbon of the arginine was isolated as barium carbonate after treatment of the arginine with arginase (prepared according to Hunter and Downs (16)), and digestion of the liberated urea with urease. No effort was made to purify the arginine flavianates since arginase exerts selective action on the arginine. The atom per cent excess of C^{13} in the arginine of liver was significantly above normal (Table III).

TABLE III

C^{13} *Concentration of Amino Acids*

	Glutamic acid	Aspartic acid	Arginine
	α-COOH carbon	Both COOH carbons	Amidine carbon
	atom per cent excess	atom per cent excess	atom per cent excess
Liver..	0.06	0.04	0.09
Kidney-spleen................................	0.02	0.00	0.03
Muscle..	0.03	0.00	0.00

The decarboxylation of the glutamic acid by ninhydrin yields carbon dioxide from the carboxyl group α to the amino group. The ninhydrin treatment of aspartic acid removes both carboxyls.

DISCUSSION

One of the chief reactions for the incorporation of carbon dioxide into the tissues of an animal involves carboxylation of pyruvate to form oxalacetate and subsequent formation of α-ketoglutarate containing the assimilated carbon. Wood *et al.* and Evans *et al.* have demonstrated, in experiments with liver slices, the presence of isotope in the α-ketoglutarate molecule, and proof has been provided that the labeled position is the carboxyl carbon α to the keto group. In recent studies by Utter and Wood (17) on pigeon liver slices, there is definite evidence that fixation of carbon dioxide occurs at the carboxyl group adjacent to the methylene carbon in oxalacetate. It is to be expected that amino acids will be formed from these two acids since oxalacetate and α-ketoglutarate can be aminated to aspartate and glutamate, respectively (18). One should, therefore, expect isotopic carbon to occur in a carboxyl group of the aspartic acid and in the α-carboxyl group of the glutamic acid. The data in Table III show this to be true.

Additional evidence for carbon dioxide incorporation is afforded by the presence of isotope in the amidine carbon of the arginine of the liver protein. This fact, taken in conjunction with the occurrence of labeled carbon in the urea molecule, supports the hypothesis of Krebs that arginine may be a participant in the cyclic series of reactions whereby urea is formed from CO_2, NH_3, and ornithine. An isotope concentration 4 times as great in the urea isolated from the urine as in the amidine carbon of the liver arginine might appear to support the argument that the ornithine plays only a minor rôle in urea formation (19). However, the arginine isolated was from the liver protein and was not the "free" arginine thought to be active in the cycle.

Incorporation of C^{13} in the carbonate of the bone in equal concentrations in the epiphyseal, subepiphyseal, and diaphyseal regions may indicate an adsorption of calcium carbonate rather than an exchange reaction.

SUMMARY

Hourly intraperitoneal injections of an adult female rat with isotopic sodium bicarbonate containing excess C^{13} over a period of 18 hours resulted in an incorporation of labeled carbon in bone, urea, the amidine carbon of the arginine, the α-carboxyl group of glutamic acid, and in a carboxyl group of aspartic acid. The presence of isotope in the amidine carbon of the arginine provides support for the occurrence of the Krebs ornithine cycle *in vivo*. The position of the isotope in the dicarboxylic amino acids is in agreement with what would ` ɔ expected from carbon dioxide assimilation studies of liver slices ir   ʌ.

There is definite evidence piovided that assimilated carbon dioxide finds its way into the amino acids of the body protein.

BIBLIOGRAPHY

1. Wood, H. G., and Werkman, C. H., *Biochem. J.*, **30**, 48 (1936).
2. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 365, 377, 483 (1941).
3. Ruben, S., and Kamen, M. D., *Proc. Nat. Acad. Sc.*, **26**, 418 (1940).
4. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 301 (1940); **141**, 439 (1941).
5. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 483 (1941); **142**, 31 (1942).
6. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).
7. Lorber, V., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **151**, 647 (1943).

8. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140,** 171 (1941).
9. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159,** 475 (1945).
10. Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136,** 799 (1940).
11. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136,** 805 (1940).
12. Wood, H. G., Vennesland, B., and Evans, E. A., Jr., *J. Biol. Chem.*, **159,** 153 (1945).
13. Steenbock, H., *Science*, **58,** 449 (1923).
14. Sumner, J. B., and Sisler, E., *Arch. Biochem.*, **4,** 207 (1944).
15. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141,** 627 (1941).
16. Hunter, A., and Downs, C. E., *J. Biol. Chem.*, **155,** 173 (1944).
17. Utter, M. F., and Wood, H. G., *J. Biol. Chem.*, **160,** 375 (1945).
18. Braunstein, A. E., and Kritzmann, M. G., *Enzymologia*, **2,** 129 (1937).
19. Bach, S. J., Crook, E. M., and Williamson, S., *Biochem. J.*, **38,** 325 (1944).

THE DETERMINATION OF SERUM PHOSPHATE BY THE MOLYBDIVANADATE METHOD

By DAISY G. SIMONSEN, MAXINE WERTMAN, LEOLA M. WESTOVER, AND JOHN W. MEHL

(From the Departments of Medicine and Biochemistry, School of Medicine, University of Southern California, and the Los Angeles County General Hospital, Los Angeles)

One of the commonly used methods for the colorimetric determination of phosphorus is based upon the reduction of molybdic acid to molybdenum blue and subsequent estimation of the intensity of the blue color formed. An excellent discussion of the molybdenum blue reaction and its limitations is given by Woods and Mellon (1). In using this reaction over a period of years the authors have been increasingly aware of its shortcomings.

Acid stannous chloride was used as the reducing agent and the procedures of Kuttner and Cohen (2), Woods and Mellon (1), and Fontaine (3) were carefully followed. In addition, modifications in concentration and type of both acid and molybdate have been studied. The results obtained were in accord with those of Woods and Mellon (1) who found that the relative amounts of acid and molybdate were important since, if the ratio of acid to molybdate was too low, some of the molybdate was reduced along with the heteropoly acid, and if too high, there was a marked decrease in the intensity of the color. In general, the difficulties encountered in our laboratory with the use of chlorostannous acid as the reducing agent were (1) control of concentration of acid and molybdate, (2) instability of the color after a short time interval, (3) non-conformity with Beer's law when the concentration of phosphorus was above 4 parts per million, (4) rapid deterioration of the stannous chloride solution, particularly of the dilute reagent, and (5) inability to obtain duplicate results from day to day when standard solutions of phosphate were used.

Three recent methods for the determination of phosphate have made use of ammonium vanadate with subsequent colorimetric estimation of the yellow molybdivanadophosphoric acid formed. Willard and Center (4) described a method for the determination of phosphorus in iron ore, Koenig and Johnson (5) one for the determination of phosphorus in foods and biological material, and Kitson and Mellon (6) a method used for the determination of phosphorus in carbon and low alloy steels. Since the procedure seemed to offer distinct advantages from the standpoint of stability of the final color and simplicity, the application of the method of Kitson and Mellon to the determination of serum phosphate has been investigated.

747

748 DETERMINATION OF SERUM PHOSPHATE

Procedure

Reagents—

1. Redistilled water (from an all-glass still) or distilled water known to be phosphate-free. This water was used for the preparation of all reagents.

2. Trichloroacetic acid, 7.5 and 10 per cent solutions.

3. Ammonium molybdate, 5 per cent aqueous solution.

4. Ammonium vanadate,[1] 0.25 per cent solution. Dissolve 2.5 gm. of ammonium vanadate in approximately 500 ml. of boiling water, cool slightly, and then add 20 ml. of concentrated nitric acid. Allow the mixture to cool to room temperature and make up to a volume of 1 liter.

5. Nitric acid (1:2);[2] 1 volume of concentrated nitric acid plus 2 volumes of water.

6. Phosphate standard. Dissolve 0.4389 gm. of reagent grade potassium dihydrogen phosphate (previously dried to constant weight) in 7.5 per cent trichloroacetic acid and make to a volume of 1 liter (with the same strength acid). 1 ml. of this solution contains 100 γ of phosphorus. Suitable dilute standards (in 7.5 per cent trichloroacetic acid) are prepared from the stock solution to give a range of 5 to 80 γ of phosphorus in the final 5 ml., which was adopted as the standard volume for this procedure.

*Procedure for Klett-Summerson Photoelectric Colorimeter—*Measure into a calibrated Klett tube (preferably graduated at 5 ml.) 3 ml. of a suitable dilute standard or sample. Add 0.5 ml. of 1:2 nitric acid and mix by thorough shaking. Then add 0.5 ml. of 0.25 per cent ammonium vanadate solution and mix by shaking. Finally add 0.5 ml. of 5 per cent ammonium molybdate, make to a volume of 5 ml. with water, and mix by inversion. After the mixture has stood 5 minutes, read in the colorimeter with the No. 42 (blue) filter. A blank is prepared with 3 ml. of 7.5 per cent trichloroacetic acid and the reagents added in the same amounts and order as for the standard. In every case the calculations are based upon the reading after subtraction of the blank reading.

*Procedure for Serum—*To 6 ml. of 10 per cent trichloroacetic acid in a 15 ml. centrifuge tube add 2 ml. of serum. Mix by inversion, allow to stand 15 minutes, and then centrifuge for 7 to 10 minutes at 2500 to 3500 R.P.M. or until the supernatant liquid is clear. Measure 3 ml. of the supernatant

---

[1] May be obtained from Eimer and Amend, New York.

[2] After the larger part of the experiments had been completed, it was found that the vanadate and nitric acid could be combined by dissolving the ammonium vanadate as directed, but adding 350 ml. of concentrated nitric acid instead of 20 ml., and diluting to 1 liter. 0.5 ml. of this combined reagent is used in place of 0.5 ml. each of the original ammonium vanadate and 1:2 nitric acid. The strongly acid vanadate solution has given the same results as those obtained with the original reagents, and has been stable for the 2 months that it has been in use.

fluid (equivalent to 0.75 ml. of serum) into a calibrated Klett tube, and proceed in the same manner described for the standard solutions. The number of micrograms of phosphorus, taken from the colorimeter reading of the standard curve, multiplied by 0.133 equals the number of mg. of phosphorus per 100 ml. of serum.

Inasmuch as the nitric acid and vanadate could be combined as one reagent, the procedure for the determination of serum phosphorus was altered as follows: To 4 ml. of the trichloroacetic acid filtrate add 0.5 ml. of the combined vanadate-nitric acid reagent, then 0.5 ml. of ammonium molybdate, and mix by inversion. Read in the colorimeter after a 5 minute interval.

It is advisable to run one or two standards with each set of unknowns in order to check for contamination and stability of reagents and any variability of the colorimeter. A blank determination is also made each time.

Spectrophotometric Determinations—Spectrophotometric measurements were also made in certain instances. The determinations were made in the Beckman quartz spectrophotometer, with 1.00 cm. Corex cells. The results are reported as the observed optical densities of the solutions.

Results

Phosphate Standards—The phosphate standards were routinely made up in trichloroacetic acid of the same concentration as that in the serum filtrates, since some effect of trichloroacetic acid was noted in the higher concentrations of phosphate. The calibration curves are given in Fig. 1. The curve of corrected readings against concentration is not strictly linear when the amount of phosphate corresponds to 5 to 80 γ of P in the final 5 ml.

This deviation from a straight line is doubtless attributable to the spectral characteristics of the filter and the response of the photocell. The optical densities of these same solutions, measured at 440, 420, 400, and 380 mμ, are given in Fig. 2. In each case the absorption of the blank at the corresponding wave-length has been subtracted. The solutions will be seen to follow Beer's law.

It is also evident that the absorption increases with decreasing wave-length. Since it was not clear from previous reports whether there might be an absorption peak which could be used for spectrophotometric studies, the values of the optical densities of the blank and of solutions containing 0.1 and 0.2 mg. of P per 100 ml. were determined at several wave-lengths and are given in Fig. 3. It was not possible to determine the position of the maximum because of the very great absorption of the blank at the shorter wave-lengths, but it is evident that there is little to be gained by using wave-lengths below about 380 mμ, since the blank is increasing much more rapidly than the value of $E_{1\ cm.}^{1\%}$.
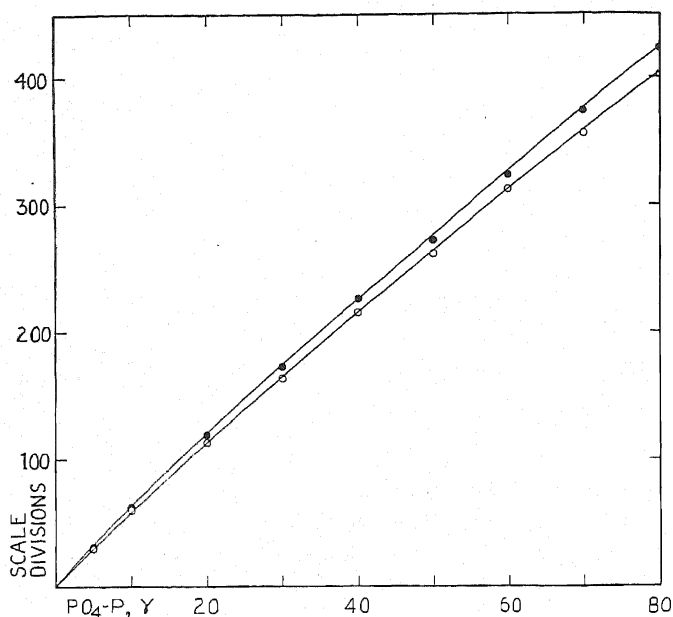
FIG. 1. Curves of Klett-Summerson colorimeter readings (corrected for the blank) for various amounts of phosphate P in the final 5 ml. of solution. ○, phosphate solutions in water; ●, phosphate solutions in 7.5 per cent trichloroacetic acid.
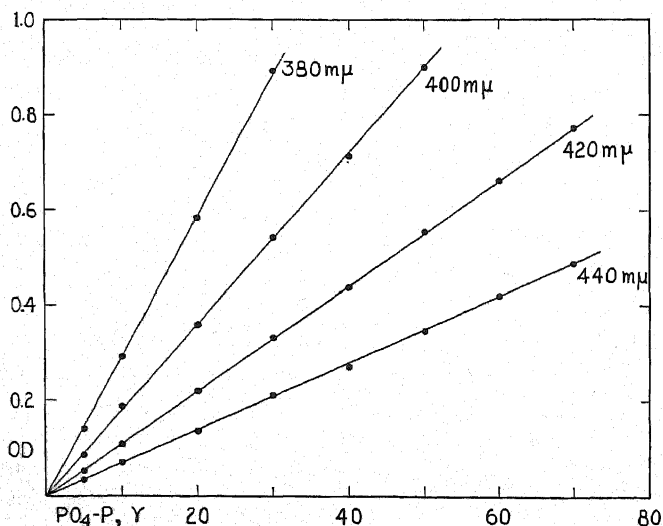
FIG. 2. Optical densities of solutions (corrected for the blank) for various amounts of phosphate in the final 5 ml. of solution, at 440, 420, 400, and 380 mμ. All phosphate solutions were made in 7.5 per cent trichloroacetic acid.

Some attention was given to the possibility of reducing the blank by decreasing the concentration of the reagents employed.   This attempt was not successful, since the high blank is largely due to the molybdate, and color development does not take place in the absence of the large excess of molybdate specified in the procedure.

Although greater sensitivity can be obtained with the spectrophotometer, the results to be presented were obtained with the colorimeter, since this instrument is more likely to be used in routine procedures.
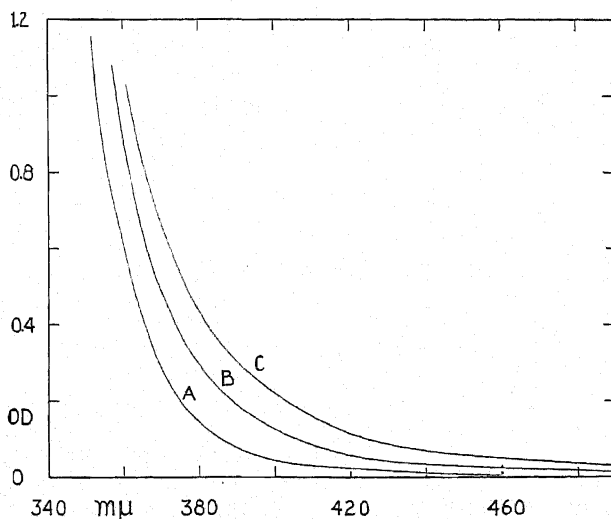
FIG. 3. Optical densities of the blank (Curve A), a solution containing 0.1 mg. of PO_4-P per 100 ml. (Curve B), and of a solution containing 0.2 mg. of PO_4-P per 100 ml. (Curve C), as a function of the wave-length.

The color of the blanks and the test solutions is entirely reproducible and is stable for periods of at least 24 hours.   Duplicate determinations did not vary more than 3 divisions on the colorimeter scale, and were usually in perfect agreement.   The blank gives a reading of 10 to 11 divisions, and the increase over the blank is 26 to 30 divisions per microgram of P per ml., depending upon the concentration.

After the color has been developed, it is not possible to dilute the samples with water or 7.5 per cent trichloroacetic acid in order to obtain a lower colorimeter reading.   Readings of the diluted samples give a positive deviation, of as much as 12 per cent in the higher concentrations, when the dilution factor is taken into account.   Thus, if the unknown solution contains too high a concentration of phosphorus to be calculated from the calibration curve, one must repeat the procedure with a smaller sample.

TABLE I

Recovery of Phosphate Added to Serum

P added for each 3 ml. filtrate	P found in 3 ml. filtrate	P calculated in 3 ml. filtrate	Recovery
γ	γ	γ	*per cent*
0	41.3		
2.5	43.5	43.8	99.3
5.0	46.5	46.3	100.4
7.5	48.9	48.8	100.2
10.0	51.2	51.3	99.8
12.5	54.3	53.8	100.9
15.0	56.9	56.3	101.0
17.5	58.4	58.8	99.3
22.5	64.0	63.8	100.3
25.0	66.4	66.3	100.1

Average error . . . . ±0.5

TABLE II

Serum Phosphate Values of Normal Adults

Patient No.	P per 100 ml. serum	
	Females	Males
	mg.	*mg.*
1	4.1	3.2
2	3.6	3.1
3	4.0	3.6
4	4.9	4.3
5	3.9	3.5
6	4.6	3.9
7	3.9	3.0
8	4.3	2.8
9	3.9	4.3
10	4.4	3.6
11	4.0	3.3
12	3.5	4.3
13	4.0	3.6
14	3.9	4.5
15	4.1	4.8
16	3.9	4.5
17	4.5	3.5
18	3.7	4.3
19	3.6	4.0
20		3.3
21		3.9
Average	4.0	3.8

Recovery of Phosphate Added to Serum—Various phosphate standards were added to 3 ml. samples of pooled serum so that the added phosphorus ranged from 10 to 100 γ per 3 ml. of serum. Sufficient water and 20 per cent trichloroacetic acid were then added so that the final volume and acidity were identical with those used in the determination of serum phosphate.

TABLE III

Serum Phosphate Values of Children

Patient No.	Sex	Age	P per 100 ml. serum	Diagnosis
		yrs.	*mg.*	
1	F.	11	5.5	Controlled diabetes
2	"	3	5.6	Probable infectious mononucleosis
3	M.	13	5.9	Acute rheumatic fever
4	"	13	7.2	" " "
5	"	13	5.9	" " "
6	"	9	5.1	" " "
7	F.	12	5.1	"Streptococcus" throat
8	M.	10	5.1	Sickle cell anemia
9	"	13	7.1	Probable subacute glomerulonephritis
10	"	9	7.2	" " "
11	"	9	6.0	Acute glomerulonephritis
12	F.	11	5.1	Undiagnosed
13	"	12	6.4	" but possible anemia
14	"	8	5.3	Cellulitis
15	"	11	5.2	Possible rheumatic fever
16	"	11	5.1	" " "
17	"	10	4.6	Mumps or recurrent parotitis
18	"	9	5.9	Upper respiratory infection
19	"	11	4.9	Tonsillitis
20	"	7	5.6	Lobar pneumonia
21	"	9	6.1	" "
22	"	12	6.7	Acute glomerulonephritis
23	"	12	5.7	Rheumatic fever
Average			5.7	

The phosphorus was then determined in 3 ml. of the acid filtrate according to the standard procedure. The recoveries are given in Table I.

The results show an excellent recovery of added phosphorus. The errors are no greater than those that would be obtained by setting up and reading a series of different sera or a set of standards, and give an average deviation of ±0.5 per cent from the calculated value.

Serum Phosphate of Normal Adults—Blood samples were obtained from members of the laboratory staff which included chemists, physicians, technicians, medical students, and general laboratory assistants. There were

forty adults in the group, ranging in age from 20 to 60 years. Sex distribution showed nineteen females and twenty-one males. The values obtained ranged from 2.8 to 4.9 mg. of phosphorus per 100 ml. of serum. The results are given in Table II.

Serum Phosphate of Children—A group of twenty-three hospitalized children was used for the study of phosphorus values of children. Patients with bone disease were excluded; otherwise the cases were selected at random. Since the majority were near the end of convalescence and ready to be dismissed from the hospital, the values approximate those of normal children. The values obtained range from 4.6 to 7.2 mg. of phosphorus per 100 ml. of serum. The difference between the lowest values of children and adults was 1.8 mg. and that of the highest values was 2.3 mg. of P per 100 ml. of serum. Based on the average values of the two groups, the children had a serum phosphate higher by 1.8 mg. of P per 100 ml. of serum than the adults. The complete results are given in Table III.

<center>DISCUSSION</center>

The method is based upon the yellow color formed when an excess of molybdate is added to an acidified solution of an orthophosphate and a vanadate. The exact nature of the reaction is uncertain, but presumably depends upon the formation of a heteropoly complex, $(NH_4)_3 \cdot PO_4 \cdot NH_4VO_3 \cdot 16MoO_3$, such as that formulated by Mission (7). This method is less sensitive than the molybdenum blue methods, but is adequate for most biological applications, and has the great advantage of providing a stable solution for colorimetry. We feel that this advantage, together with a lower sensitivity to changes in final acid concentration, outweighs the advantages of the molybdenum blue methods for orthophosphate determinations.

<center>SUMMARY</center>

1. The molybdivanadate method for orthophosphate, as described by Kitson and Mellon, is suitable for the determination of serum phosphate.

2. The solutions obey Beer's law, and the method may be used spectrophotometrically for concentrations of PO_4-P between 0.5 and 16 γ per ml.

3. The increase in colorimeter readings was not found to be strictly proportional to the concentration of phosphate, but this appeared to be due to the characteristics of the instrument. The method is applicable to the photoelectric colorimeter for the range of concentrations from 2 to 16 γ of P per ml.

4. The serum phosphate in twenty-one normal, adult males was found to average 3.8 mg. of P per 100 ml., with an extreme range of 2.8 to 4.8 mg. per 100 ml. The values in nineteen females averaged 4.0 mg. per 100 ml., with a range from 3.5 to 4.9 mg. per 100 ml.

5. The serum phosphate in twenty-three children had an average value of 5.7 mg. of P per 100 ml., and varied from 4.6 to 7.2 mg. per 100 ml.

BIBLIOGRAPHY

1. Woods, J. T., and Mellon, M. G., *Ind. and Eng. Chem., Anal. Ed.,* **13,** 760 (1941).
2. Kuttner, T., and Cohen, H. R., *J. Biol. Chem.,* **75,** 517 (1927).
3. Fontaine, T. D., *Ind. and Eng. Chem., Anal. Ed.,* **14,** 77 (1942).
4. Willard, H. H., and Center, E. J., *Ind. and Eng. Chem., Anal. Ed.,* **13,** 81 (1941).
5. Koenig, R. A., and Johnson, C. R., *Ind. and Eng. Chem., Anal. Ed.,* **14,** 155 (1942).
6. Kitson, R. E., and Mellon, M. G., *Ind. and Eng. Chem., Anal. Ed.,* **16,** 379 (1944).
7. Mission, G., *Chem. Ztg.,* **32,** 633 (1908).

CHROMOGENIC SUBSTRATES

II. PHENOLPHTHALEIN GLUCURONIC ACID AS SUBSTRATE FOR THE ASSAY OF GLUCURONIDASE ACTIVITY*

By PAUL TALALAY, WILLIAM H. FISHMAN, AND CHARLES HUGGINS

(*From the Departments of Surgery and Biochemistry, University of Chicago, Chicago*)

Increasing interest in the enzyme hydrolyzing naturally occurring and synthetic β-glucuronides has shown the need for a more satisfactory method of assay for β-glucuronidase. The present paper describes the use of phenolphthalein mono-β-glucuronide as a chromogenic substrate for the assay of the hydrolytic activity of β-glucuronidase. The phenolphthalein mono-β-glucuronide is prepared biosynthetically from the urine of rabbits to which phenolphthalein has been administered. This substrate is rapidly hydrolyzed by β-glucuronidase, and the free phenolphthalein may be readily determined photocolorimetrically in alkaline solution. The method of assay is similar in principle to the use of sodium phenolphthalein phosphate in the measurement of phosphatase activity (1).

Definition of Enzyme—Masamune (2) first defined the enzyme splitting the glucuronide linkage as a "glucuronosidase." He found that biosynthetic menthol and phenol glucuronic acids are hydrolyzed very slowly by emulsins, and that ox kidney extracts have a powerful hydrolytic effect on β-glucuronides. Masamune demonstrated the hydrolysis of borneol, β-naphthol, menthol, phenol, and phloroglucinol glucuronic acids. Synthetic α-glucosides and α-menthol glucuronic acids were not split by these enzyme extracts, while only some β-glucosides other than β-glucuronides are attacked. Fishman (3, 4) found also that extracts of various animal tissues caused the hydrolysis of borneol and menthol glucuronic acids. Estriol glucuronic acid is split by the same enzyme extracts, which have been termed preparations of "β-glucuronidase."

Review of Methods—Masamune (2) and Oshima (5) assayed the activity of the enzyme, using the Hagedorn and Jensen method for the measurement of the reducing power. The difference in reducing power between controls containing inactivated enzyme and experimental tubes is taken as a measure of the amount of hydrolysis of the glucosidic linkage and the formation of free glucuronic acid. For borneol and menthol glucuronides the method

can be applied directly to the protein-free filtrates. When β-naphthol and phenol glucuronides are used, the ferricyanide is reduced not only by the free glucuronic acid, but also by the free phenols, and these must be removed by extraction before the reducing power of the residual solutions is measured.

In a reexamination of the assay procedure, Fishman (3) substituted a modification of the ceric sulfate titration of Miller and Van Slyke (6), using sodium menthol glucuronic acid (3) and borneol glucuronic acid (4) as substrates; the reducing powers were compared by means of a standard curve of pure glucurone. In studies on the kinetics of the hydrolysis of estriol glucuronic acid, Fishman (4) separated free estriol liberated from estriol glucuronic acid by the enzyme, and determined this estriol by the colorimetric method of Venning, Evelyn, Harkness, and Browne (7). The use of estriol glucuronic acid as a substrate is not practicable, since the material is difficult to obtain.

Limitations of Methods—Two principal factors have limited the accuracy and reliability of the assay of glucuronidase activity by measuring the increase in reducing power resulting from the splitting of the β-glucosidic linkage of glucuronides and the liberation of free glucuronic acid. (1) In the determination of the activity of tissue extracts or biological fluid, reducing substances other than glucuronic acid are often present in relatively high amounts, and for reliable results the increment in reducing power produced by enzymatic action must be large compared to the control. This necessitates the use of very long incubation times, or of large amounts of enzyme. Moreover, the determination depends on the difference between two measurements, and errors are thus magnified. (2) The production of non-glucuronic acid reducing substances in the reaction mixture, due to the presence of enzymes other than glucuronidase in tissue extracts, cannot be excluded; this will not occur in the controls if these contain heat-inactivated enzymes. If the controls are set up without substrate but contain the active enzyme extract, this may act on preformed natural glucuronides present in the tissue extracts and thus increase the reducing power of the controls.

Thus, to illustrate the problem, there is a measurable increase in reducing power on incubation of plasma in the absence of added substrate.[1] This may be due to the breakdown of substances yielding reducing power, or to the action of enzymes other than glucuronidase on substrates in the serum or to the action of glucuronidase on glucuronides present in the serum. These alternatives cannot always be distinguished by careful controls, especially when the glucuronidase activity is small.

[1] Fishman, W. H., unpublished.

Quantitative methods for glucuronic acid, such as a spectrophotometric modification of the Tollens naphthoresorcinol reaction (8), cannot be readily applied to the measurement of glucuronidase activity, since, under the conditions of the test, strong hydrochloric acid hydrolyzes the conjugated glucuronides, and does not distinguish between free and conjugated glucuronic acid.

The need exists for a method of assaying glucuronidase activity, depending either upon a specific quantitative reaction distinguishing free and combined glucuronic acid or upon a satisfactory assay of the nonglucuronic acid part of the substrate molecule, with a minimum of interference from naturally occurring substances. Such a method should be sensitive and eliminate long hours of incubation, with the consequent objections of decay in reaction velocity and destruction of the enzyme and bacterial action in the reaction mixture.

Principles of Present Method—Phenolphthalein mono-β-glucuronide was described by Di Somma (9) as appearing in the urine as the product of the conjugation of administered phenolphthalein in rabbits; he noted that this compound was hydrolyzed to a small extent by crude almond emulsin, while crude kidney extract showed a strong hydrolytic action, evidently due to the presence of β-glucuronidase.

Examination of the absorption spectra of phenolphthalein and its monoglucuronide was undertaken with a view of utilizing the glucuronide as a substrate for the assay of β-glucuronidase activity. Both compounds appear colorless in the acid range. Phenolphthalein exhibits its maximum stable color intensity at pH 10.0 to 10.4 (1). The monoglucuronide is yellow in alkaline solution. The absorption spectra of the two compounds in the visible range were compared in 0.1 M glycine buffer, pH 10.2, with a Beckman model DU spectrophotometer. The absorption curves are plotted in Fig. 1, in which the extinction coefficients of the monoglucuronide are shown on a scale 200 times larger than the coefficients of phenolphthalein. The principal maximum of phenolphthalein is at 550 to 555 mμ (ϵ = 26,600 liters per mole $\times$ cm.) and the maximum of phenolphthalein mono-β-glucuronide lies between 415 and 420 mμ (ϵ = 130 liters per mole $\times$ cm.).

The mono-β-glucuronide has an extinction coefficient of 48 liters per mole $\times$ cm. at 552 mμ, which is the maximum of phenolphthalein, so that at the absorption peak of phenolphthalein, the monoglucuronide has only 0.18 per cent of the absorption of the free phenolphthalein for the same concentration of the two compounds.

These profound differences in degree and position of absorption maxima of phenolphthalein and its glucuronide make it possible to use phenolphthalein mono-β-glucuronide as a substrate for the assay of β-glucuronidase activity by measuring free phenolphthalein colorimetrically near its absorp-

tion maximum.   In this assay procedure, the monoglucuronide is in part converted to free phenolphthalein, and the removal of the absorption due to the monoglucuronide and its replacement by free phenolphthalein will only introduce an error of 0.2 per cent compared to the control if measured at 550 mμ.

Thus, if 5 per cent of phenolphthalein monoglucuronide is hydrolyzed in an experiment with the production of an equivalent amount of free phenolphthalein, and no hydrolysis occurs in the controls, the comparison of the absorption of control and experimental tubes at 550 mμ will not measure the
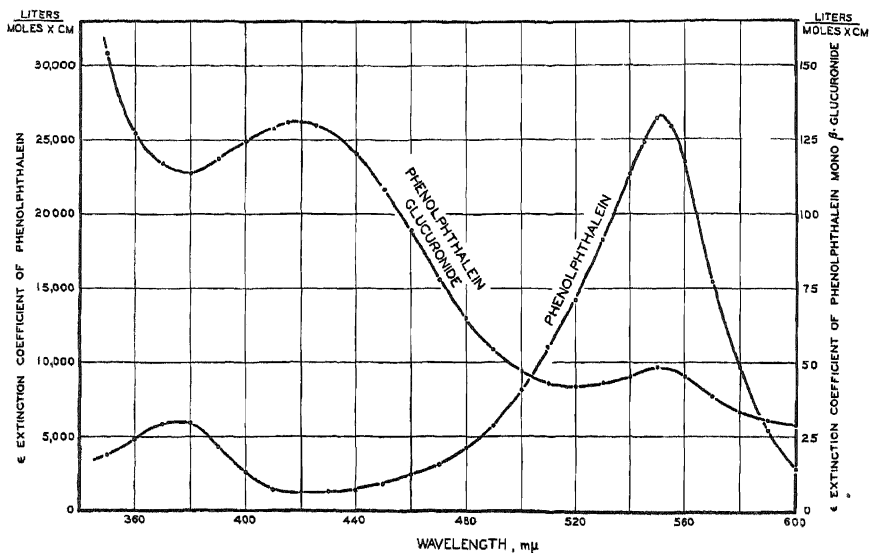
FIG. 1. Spectrophotometric absorption curves of phenolphthalein and phenolphthalein mono-β-glucuronide in 0.1 M glycine buffer, pH 10.2.   The scale for the extinction coefficients of the glucuronide is magnified 200 times relative to that of phenolphthalein.

true amount of phenolphthalein, since the control contains 5 per cent more monoglucuronide than the experimental.   The liberated phenolphthalein absorbs under these conditions 500 times as strongly as the equivalent amount of monoglucuronide and the deviation from the true phenolphthalein measurement is negligible and does not constitute a limiting factor in the accuracy of the method.

Isolation of Phenolphthalein Glucuronide

Di Somma (9) studied the fate of subcutaneously and intramuscularly administered colloidal phenolphthalein in rabbits and guinea pigs.   He

obtained both free and combined phenolphthalein from the urine of such animals. From the urine of rabbits Di Somma isolated phenolphthalein mono-β-glucuronide and crystallized this as the cinchonidine salt.

Phenolphthalein mono-β-glucuronide was prepared from rabbit urine by a modification of Di Somma's procedure which permitted the isolation of larger quantities of the compound over a prolonged period of time. Rabbits were kept in a metabolism cage (four or five in a large cage) on a diet of carrots with adequate water intake. The phenolphthalein was adminis- tered as a water-soluble derivative. Daily subcutaneous injections of an aqueous solution of 500 mg. of sodium phenolphthalein phosphate (1)[2] were given to each rabbit, and the urine was collected and measured daily. A little toluene was added as a preservative. 10 liter batches of urine were worked up at a time (the average output of each rabbit was 150 to 250 ml. in 24 hours). The deeply colored urine was filtered through coarse fluted paper to remove gross solid particles. The red color of free phenolphthalein became evident in the alkaline urine (pH 8 to 9) during the 1st day after the initial injection.

The administration of phenolphthalein in the form of its water-soluble phosphate was found to be more convenient than the use of colloidal solutions. 2 per cent colloidal gelatin suspensions of phenolphthalein, as used by Di Somma, are absorbed slowly, cannot be administered in large quantities, are tedious to prepare, and remain under the skin for long periods, while the concentrated water solutions of phenolphthalein phos- phate can be administered conveniently and simply in large amounts.

To the urine, after filtration, were added 0.1 volume of 30 per cent sodium chloride and then 0.1 volume of 3 per cent colloidal ferric hydroxide. (The colloidal ferric hydroxide is readily prepared by pouring 100 ml. of 30 per cent aqueous ferric chloride solution into 900 ml. of boiling distilled water.) The addition of ferric hydroxide to the urine produced a flocculent brown precipitate which was permitted to settle and filtered off. In this way most of the proteins were precipitated and some of the coloring matter removed. Precipitation with 3 volumes of acetone as recommended by Di Somma was abandoned since the large volumes become hard to handle and the subse- quent evaporation of the acetone is time-consuming.

The free phenolphthalein was then removed from the urine by extraction with ethyl ether. 3 liters of urine were extracted at a time in a 5 liter separatory funnel, with four 300 to 400 ml. ether portions. The ether ex- tract gave positive tests for free phenolphthalein with alkali, while the aqueous phase was almost free of it. The aqueous solution was acidified with concentrated hydrochloric acid until blue to Congo red (pH 4 to 5)

[2] The sodium phenolphthalein phosphate was supplied by Paul Lewis, Inc., 918 North Fourth Street, Milwaukee, Wisconsin.

and was extracted with four portions of ethyl acetate (300 to 400 ml. each). The ethyl acetate layer formed a foamy gel which was separated and permitted to filter by gravity through a fluted filter paper. The filtration broke the mucilagenous emulsion and formed clear layers, so that additional amounts of the aqueous phase could be separated off and discarded. The pooled ethyl acetate extracts were reduced to a small volume *in vacuo* with slight heating. The amber-brown liquid (about 200 ml., representing an extract of 10 liters of urine) was decolorized by shaking with activated charcoal (Merck) and then filtered. The light yellow solution so obtained was mixed with an excess of a saturated ethyl acetate solution of cinchonidine (the free alkaloid of Merck), whereupon a white or slightly yellow precipitate formed. The suspension was left in the ice box overnight and the precipitate filtered off in the morning. It was washed with ethyl acetate and anhydrous ethyl ether and represents the crude product (cinchonidine salt of phenolphthalein glucuronide). The filtrate was reconcentrated under a vacuum and on the addition of more saturated cinchonidine solution yielded further product. This was repeated once more to obtain more material.

The crude cinchonidine salt was repurified by crystallization from hot dioxane. Best results were obtained by dissolving the salt in boiling dioxane (redistilled Eastman Kodak dioxane) in which it is completely soluble and adding hot ethyl acetate until slight turbidity results. The cinchonidine salt crystallized on slow cooling as pure white sheaves and rosettes of needles. All products were recrystallized at least twice.

The compound was dried in a vacuum desiccator over phosphorus pentoxide. It was analyzed for hydrolyzable phenolphthalein. A weighed amount of compound was refluxed with 4 N HCl for 1 hour, and the solution was neutralized and made up to a standard volume with water. A small aliquot was mixed with glycine buffer, pH 10.2, and compared with phenolphthalein standards. 38.1 per cent of phenolphthalein was found, which may be compared with 40.4 per cent calculated for pure phenolphthalein glucuronide and 36.3 per cent calculated for this compound containing 1 mole of dioxane of crystallization. Evidently this compound is phenolphthalein monoglucuronic acid, possibly containing solvent of crystallization. Free phenolphthalein was not present and no traces of either phosphate or sulfate could be detected. These experiments show that water-soluble sodium phenolphthalein phosphate administered subcutaneously is excreted in large part as the monoglucuronide, which may then be obtained in pure form as the cinchonidine salt.

The yield of crude cinchonidine salt (prior to recrystallization) represents at least a 25 per cent conjugation of the injected phenolphthalein. The crude material may contain small quantities of phenolphthalein phosphate but these are completely eliminated in the recrystallizations.

If the collection of urine is continued beyond the last administration of phenolphthalein phosphate, free phenolphthalein is apparent in the urine for some 2 weeks, but no conjugate is present 3 or 4 days after the last injection. In a typical experiment five rabbits were injected with 26.5 gm. of sodium phenolphthalein phosphate over a period of 17 days and the urine collected for 21 days. 16 liters of urine obtained during this period yielded 8.26 gm. of the crude cinchonidine salt. Urine beyond the 21st day continued to give a strong reaction for free phenolphthalein, but contained almost no conjugate.

Kinetics of Hydrolysis of Phenolphthalein Mono-β-glucuronide—The kinetics of the enzymatic hydrolysis of phenolphthalein glucuronide were investigated with a glucuronidase preparation obtained from the livers, kidneys, and spleens of white mice.

Preparation of β-Glucuronidase—The enzyme was prepared by the rapid method of Fishman.[1] Pooled spleens, livers, and kidneys of a dozen mice were rapidly homogenized in a Waring blendor and taken up in a convenient volume of chilled water (about 200 ml.). The mixture was adjusted to pH 5 with 1 N acetic acid and incubated at 37° for about half an hour; this produced flocculation of many proteins which were then removed together with the insoluble residue by centrifugation, leaving a pale red, clear supernatant which was decanted from the residue. An equal volume of saturated ammonium sulfate was added to the solution and the mixture centrifuged rapidly (3000 R.P.M.) for 30 minutes. The supernatant was removed by suction, and the residue dissolved in 25 ml. of distilled water. The enzyme solution so obtained was centrifuged and the residue discarded.

The precipitation with ammonium sulfate at half saturation may be repeated several times, but usually one more precipitation provides a strong glucuronidase preparation which is suitable for the study of the kinetics.

Optimum pH—The optimum pH of the hydrolysis of this substrate was determined in 0.2 M acetate buffer between pH 3.8 and 5.8 and in 0.2 M phosphate buffer between pH 5.8 and 7.0. In order to obtain an accurate measure of the phenolphthalein liberated at each pH, the digests on termination of incubation were all titrated with 0.1 N NaOH to the end-point of free phenolphthalein (about pH 8.3) and then an aliquot of each digest was diluted with 0.1 M glycine-sodium hydroxide buffer, pH 10.2, and compared with a standard phenolphthalein curve, correction being made for the change in volume due to the added sodium hydroxide. In this way the digests were all brought to identical hydrogen ion concentrations, regardless of the initial pH. All pH measurements were made with the glass electrode and a Leeds and Northrup potentiometer bridge.

The optimum pH for the hydrolysis of 0.01 M phenolphthalein monoglucuronide determined under these conditions is 4.5. Other workers have given pH 5.0 to 5.2 for menthol glucuronide (5), using a spleen enzyme and

acetate buffer.   Fishman (4) has reported pH 5.0 for the hydrolysis of 0.1 N sodium menthol glucuronide, pH 4.4 for sodium borneol glucuronide (0.01 M), and pH 4.3 for sodium estriol glucuronide (0.005 M), using an ox spleen glucuronidase preparation and an 0.1 M acetate buffer.

The pertinent part of the pH-activity curve is given in Fig. 2 and shows that the activity falls off somewhat more rapidly on the alkaline than on the acid side of the optimum.   At pH 7.0 the enzyme exhibits only 20 per cent of its maximum activity, and shifting the pH into the alkaline range is therefore an effective method of arresting the enzymatic reaction at the desired time.
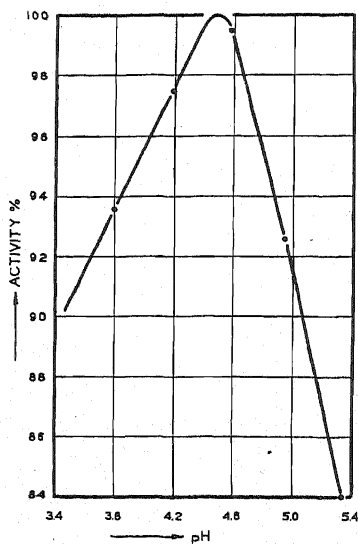
FIG. 2. The effect of pH on the activity of β-glucuronidase in 0.2 M acetate buffers, expressed as per cent of the maximum activity.

Effect of Enzyme Concentration—The hydrolysis of phenolphthalein glucuronide by serial dilutions of the enzyme showed that the amount of hydrolysis was directly proportional to the enzyme concentration, other factors being constant (Fig. 3).   This linearity was unlike the parabolic curve found by Huggins and Talalay (1) for the hydrolysis of phenolphthalein diphosphate.   It is possible that the difference lies in the fact that in the enzymatic hydrolysis of the diphosphate there is probably a differential rate of splitting off of the first and the second phosphate groups, so that the reaction is complicated by the presence of three species of compounds in the mixture (phenolphthalein monophosphate, diphosphate, and free phenolphthalein).   The linearity of the enzymatic hydrolysis of phenolphthalein glucuronide and the amount of the phenolphthalein formed is therefore

probably due to the fact that the substrate is entirely a monoglucuronide which produces free phenolphthalein on hydrolysis.

Influence of Substrate Concentration on Activity of β-Glucuronidase—The description of the kinetics of β-glucuronidase with respect to substrate
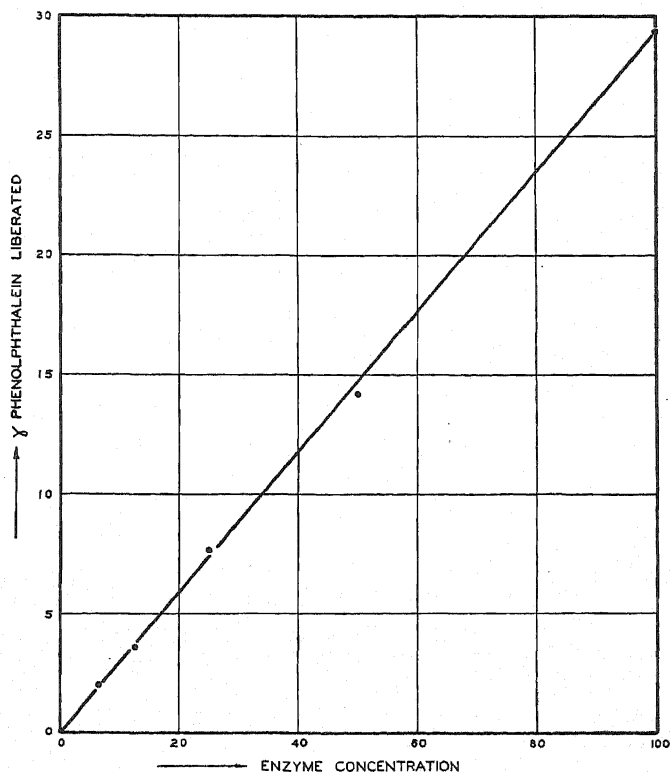
Fig. 3. The effect of enzyme concentration on the rate of hydrolysis of phenolphthalein mono-β-glucuronide. The system consisted of 3 ml. of 0.2 M acetate buffer, 1 ml. of 0.005 M sodium phenolphthalein glucuronide, and 1 ml. of various dilutions of glucuronidase. Incubated 1 hour at 38°; 5 ml. of 0.25 M glycine buffer added to develop the color and arrest the reaction. Read in an Evelyn colorimeter against a control; 540 mμ filter.

concentration was undertaken in order to establish the conditions of maximum velocity and to determine the Michaelis-Menten constant (10).

The velocity of the reaction was determined from the amount of phenolphthalein liberated in 1 hour; this can serve as a true index of initial reaction velocity, since the hydrolysis proceeds linearly with time over a period of many hours (see Fig. 6). A plot of reaction velocity against substrate concentration shows the typical curve described for many enzymes

(Fig. 4) and a slight inhibitory effect at high substrate concentration which amounted to 15 per cent for a 5-fold increase in substrate concentration above that at which the maximum velocity was obtained.

Plots of the reaction velocity according to Lineweaver and Burk (11) were made in order to obtain more accurate values for the Michaelis-Menten constant (Fig. 5). In two determinations with different enzyme concentrations, the Michaelis-Menten constants were 5.3×10^{-5} and 4.5×10^{-5} M
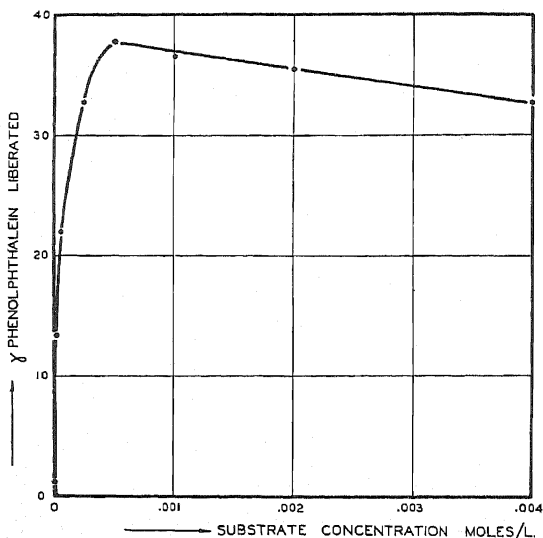
Fig. 4. The effect of substrate concentration on rate of enzymatic hydrolysis of phenolphthalein mono-β-glucuronide. The reaction rate is expressed in micrograms of phenolphthalein liberated in 1 hour at 38°. The system consisted of 4 ml. of 0.1 M acetate buffer, pH 4.5, 0.5 ml. of phenolphthalein mono-β-glucuronide, and 0.5 ml. of glucuronidase. Incubated 1 hour at 38°. Reaction stopped and color developed by addition of 5 ml. of 0.4 M glycine buffer. Color read against a control, with a 540 mμ filter.

and the maximum velocity was found to be reached at a substrate concentration of about 0.00005 M.

In our work we chose a substrate concentration of 0.001 M, which lies on the relatively flat part of the curve and at which the velocity is close to the maximum, and changes in substrate concentration of ± 10 per cent, which are limits easily reproducible, produce a change in velocity of only ± 0.5 per cent (Fig. 4).

The value for the Michaelis-Menten constant is rather lower than those reported for other substrates (4): sodium estriol glucuronide 0.0005 M, sodium menthol glucuronide 0.004 M, and sodium borneol glucuronide 0.01 M.

The rates of hydrolysis of phenolphthalein glucuronic acid and menthol glucuronic acid were compared, the same enzyme dilutions and the optimum conditions being employed for each substrate. A linear correspondence was found between the enzymatic activity of various enzyme concentrations as measured by the hydrolysis of the two substrates. In this experiment phenolphthalein mono-β-glucuronide was hydrolyzed about 2.5 times more rapidly than menthol β-glucuronide. The more rapid rate of hydrolysis of phenolphthalein glucuronide permits use of short incubation times.
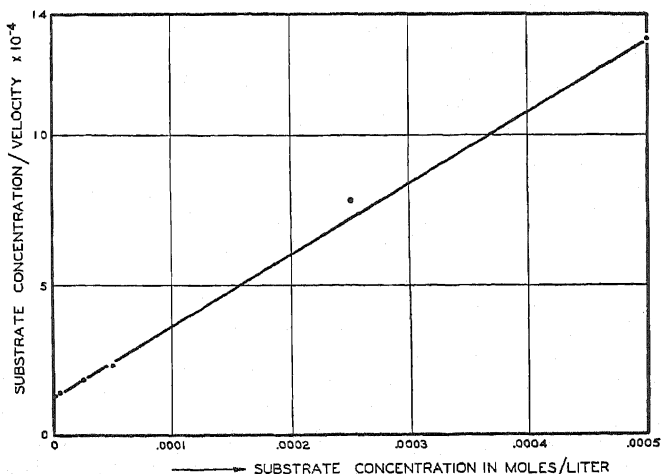
Fig. 5. The effect of substrate concentration on the rate of enzymatic hydrolysis of phenolphthalein mono-β-glucuronide. Data of Fig. 4 plotted according to Lineweaver and Burk. The velocity expressed in micrograms of phenolphthalein liberated per hour is divided by the substrate concentration in moles per liter and plotted against the substrate concentration in moles per liter. From the slope and intercept of the graph, the Michaelis-Menten constant was calculated to be 0.000053 M.

Time Course of Hydrolysis—In an experiment to determine the course of hydrolysis with time, 0.002 M phenolphthalein glucuronide in 0.1 M acetate buffer, pH 4.5, was hydrolyzed by glucuronidase for periods of time varying from 0.5 to 20 hours. This substrate concentration is sufficient to saturate the enzyme completely, so that the maximum velocity is maintained. The graph shown in Fig. 6 demonstrates that during this period of time the velocity of hydrolysis is constant, and linearity is maintained. The maximum amount of substrate decomposed at 20 hours was 9.75 per cent. The prolonged linearity of the hydrolysis indicates that the enzyme has great stability on incubation at 38° and pH 4.5, and that there was apparently no appreciable inhibition by the products of the reaction under the stated conditions.
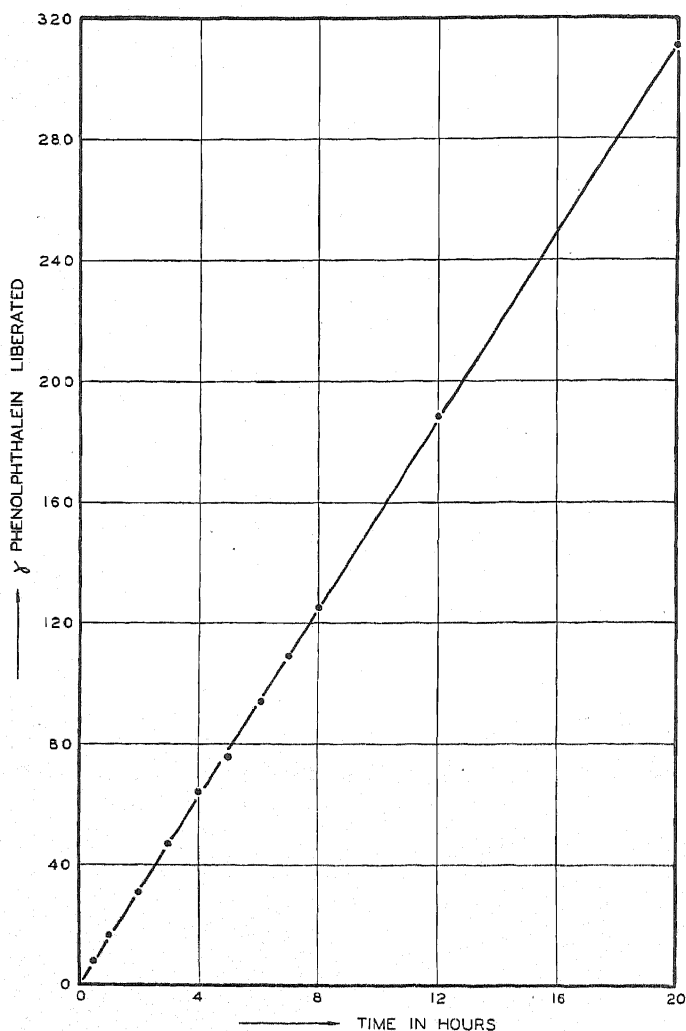
FIG. 6. The course of enzymatic hydrolysis of phenolphthalein mono-β-glucuronide in relation to time. The system consisted of 4 ml. of 0.1 M acetate buffer, pH 4.5, 0.5 ml. of 0.02 M phenolphthalein glucuronide, 0.5 ml. of glucuronidase. Incubated at 38°; color developed by addition of 5 ml. of 0.4 M glycine buffer. Readings made against controls in an Evelyn colorimeter, with a 540 mμ filter.

Optimum Conditions of Assay—The kinetic data of the action of glucuronidase on phenolphthalein mono-β-glucuronide permit definition of conditions for a routine assay of the activity of this enzyme.

The optimum rate of hydrolysis at 38° in 0.1 M acetate buffer occurs at pH 4.5. The optimum substrate concentration is in the neighborhood of 0.001 M. The time of incubation is not critical, since hydrolysis proceeds linearly with time, but the rapid hydrolysis of the substrate permits very short incubation times of 0.5 to 1.0 hours.

Reagents—

0.1 M acetate buffer, pH 4.5. 5.785 gm. of C.P. sodium acetate (CH_3-$COONa \cdot 3H_2O$) are dissolved in distilled water, 3.25 ml. of glacial acetic acid (99 per cent CH_3COOH) are added, and the mixture is made up to 1 liter with distilled water. pH 4.50. Store in the ice box.

0.01 M phenolphthalein glucuronic acid. 0.788 gm. of the pure cinchonidine salt of phenolphthalein monoglucuronide (mol. wt. 788) are mixed with an excess of approximately 2 N hydrochloric acid in a separatory funnel and the mixture extracted with several portions of C.P. ethyl acetate. The combined ethyl acetate extracts are evaporated *in vacuo* with slight warming. All traces of solvent are removed. The residue is the free phenolphthalein glucuronic acid. This is neutralized by the addition of 0.1 N sodium hydroxide and made up to 100 ml. with distilled water. It dissolves to give a slightly turbid solution which is very nearly 0.01 M with respect to phenolphthalein glucuronic acid. This amount of substrate is sufficient for about 60 determinations in duplicate. Store in the ice box.

0.4 M glycine buffer, pH 10.45 (12). This reagent arrests the reaction and develops the color. Aminoacetic acid (Merck), 16.30 gm., and sodium chloride, 12.65 gm., are dissolved in distilled water, 10.9 ml. of concentrated NaOH (100 gm. of NaOH to 100 ml. of water) (13) are added, and the mixture made up to 1 liter.

Phenolphthalein standard. 100 mg. of phenolphthalein, C.P., are dissolved in 100 ml. of 80 per cent ethyl alcohol.

Method of Performing Assay

Each determination is run in duplicate with a single control. 4 ml. of 0.1 M acetate buffer are pipetted into test-tubes, and 0.5 ml. of 0.01 M sodium phenolphthalein glucuronide is added to the two experimental tubes but not the control. The tubes are placed in a water bath at 38° and allowed to come to temperature. 0.5 ml. of enzyme solution is added to each tube at timed intervals, and the contents mixed by whirling. The tubes are stoppered and allowed to incubate for an exact period of time, usually 60 minutes. At the end of this time, 5 ml. of 0.4 M glycine buffer are added to each tube, including the control, and then 0.5 ml. of the substrate is added to the control tube. If there is any precipitation of tissue proteins in the tubes, they are centrifuged for 15 minutes at a rapid rate. The

contents of the tubes are decanted into colorimeter tubes and read in an Evelyn photoelectric colorimeter with a 540 mμ filter, the control tube in each case being set at 100. The color remains stable under these conditions of pH over a period of several hours. Controls on the spontaneous hydrolysis of the substrate have been run with each set of determinations, but the amount of hydrolysis occurring under the conditions of the test is so small as to be undetectable in the colorimeter.

The phenolphthalein calibration curve is prepared in the same buffer mixture which the experimental tubes finally contain. Colorimeter tubes are prepared to contain 4.0 ml. of 0.1 M acetate buffer at pH 4.5, 5.0 ml. of 0.4 M glycine buffer, and 1.0 ml. of phenolphthalein solution of varying dilutions. The phenolphthalein dilutions are prepared by diluting the alcohol standard (1 mg. per ml.) just prior to use. Readings are made against a water blank with a 540 mμ filter. The color is stable for many hours. A range of 5 to 60 γ of phenolphthalein per 10 ml. is conveniently covered on the Evelyn scale.

Methods of extraction of enzyme from tissues as described by Oshima (5) are tedious and involve the risk of inactivation of the enzyme by organic solvents and lengthy manipulations. Some of these undesirable features have been eliminated in a process devised by Fishman (14) for assaying glucuronidase activity in extracts of mouse organs. This method has now been adapted to the assay of the enzyme with phenolphthalein glucuronide as substrate.

In the present procedure, consistent assay on small amounts of tissue can be obtained by preparing a crude homogenate. As a check on the reproducibility of the results, a rat was bled from the carotids, and the liver removed and small pieces cut off and weighed on a torsion balance. The weighed tissue was transferred to an ice-chilled glass homogenizer, 5 ml. of chilled distilled water added, and the tissue homogenized for 1 to 2 minutes at rapid speed. The homogenate was centrifuged, the supernatant decanted, and 0.5 ml. portions assayed in duplicate for glucuronidase activity. Table I gives the values obtained for four separate samples of the same rat liver independently weighed, homogenized, and assayed in duplicate. When the activity is expressed in micrograms of phenolphthalein liberated in 1 hour at 38° per mg. of liver tissue, the values fall within ±4 per cent. The assay of solutions of the enzyme whether obtained in crude or purified state has been found to be reproducible and convenient.

Results of Assay

Some idea of the magnitude of the glucuronidase activity of various rat tissues may be obtained from Table II. No attempt is made in this paper to present average values for various tissues, but typical figures for the

TABLE I

Consistency of Assay of Glucuronidase on Samples from Single Rat Liver

Wet weight of tissue	Volume of hemogenate	Phenolphthalein liberated per tube; average of 2 determinations	Activity* of phenolphthalein per hr. per mg. wet tissue weight
mg.	*ml.*	*γ*	*units*
17.8	5	45.2	25.4
28.7	5	73.8	25.7
29.3	5	73.0	24.9
33.7	5	80.8	24.0
Average..			25.0 ± 0.7

* 1 unit of glucuronidase activity liberates 1 γ of phenolphthalein in 1 hour at 38° from phenolphthalein glucuronide at pH 4.5, under standard conditions.

TABLE II

Glucuronidase Activity of Rat Organs

Organ	Total wet weight of organ	Weight of tissue assayed	Phenolphthalein liberated per tube (10 ml.)	Activity,* per mg. wet weight of tissue	Total activity* in each organ
	gm.	*mg.*	*γ*	*units*	*units*
Liver	9.048	34.7	55.8 55.8	16.0	145,000
Spleen	0.9205	33.8	85.6 85.6	25.3	23,200
Kidney (both)	1.8202	65.6	34.4 34.4	5.25	9,550
Lung	2.535	76.5	39.9 39.1	5.15	13,100
Thyroid	0.0338	33.8	21.3 22.0	6.51	220
Adrenal	0.0136	13.6	6.0 6.8	4.63	63
Prostate	0.5368	114.8	34.8 34.8	3.0	1,610
Testis (one)	2.1323	102.3	0.8 1.1	0.1	213
Seminal vesicle	1.2035	236.3	5.5 5.6	0.23	276
Skeletal muscle	0.680	680.0	7.7 7.8	0.11	75
Ovary (one)	0.0456	45.6	43.3 41.0	9.3	424
Uterus		25.5	17.6 17.3	6.8	

* 1 unit of glucuronidase activity liberates 1 γ of phenolphthalein in 1 hour at 38° from phenolphthalein glucuronide at pH 4.5, under standard conditions.

total activity and concentration of the enzyme per mg. of wet tissue weight are presented.

SUMMARY

1. Phenolphthalein mono-β-glucuronide is proposed as a substrate for the assay of the hydrolytic activity of β-glucuronidase. The liberated phenolphthalein is measured photocolorimetrically in alkaline solution.

2. Absorption spectra and extinction coefficients of phenolphthalein and phenolphthalein mono-β-glucuronide have been determined and it is shown that phenolphthalein can be readily determined colorimetrically in the presence of its glucuronide with negligible interference.

3. The isolation and purification of phenolphthalein mono-β-glucuronide from the urine of rabbits injected with water-soluble sodium phenolphthalein phosphate is described.

4. The kinetics of the hydrolysis of phenolphthalein mono-β-glucuronide by β-glucuronidase have been investigated. The optimum pH is 4.5 in 0.1 M acetate buffer at 38°. The reaction velocity is constant with time and varies linearly with enzyme concentrations in the presence of excess substrate. The Michaelis-Menten constant is relatively low, 0.00005 M.

5. A simple method of performing glucuronidase assays on biological fluids and tissue extracts is described and typical values are given for rat organs.

BIBLIOGRAPHY

1. Huggins, C., and Talalay, P., *J. Biol. Chem.*, **159**, 399 (1945).
2. Masamune, H., *J. Biochem.*, Japan, **19**, 353 (1934).
3. Fishman, W. H., *J. Biol. Chem.*, **127**, 367 (1939).
4. Fishman, W. H., *J. Biol. Chem.*, **131**, 225 (1939).
5. Oshima, G., *J. Biochem.*, Japan, **23**, 305 (1936); **20**, 361 (1934).
6. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114**, 583 (1936).
7. Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, J. S. L., *J. Biol. Chem.*, **120**, 225 (1937).
8. Deichmann, W. B., and Dierker, M., *J. Biol. Chem.*, **163**, 753 (1946).
9. Di Somma, A. A., *J. Biol. Chem.*, **133**, 277 (1940).
10. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
11. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
12. Gortner, R. A., Outlines of biochemistry, New York, 2nd edition, 123 (1938).
13. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 29 (1932).
14. Fishman, W. H., *J. Biol. Chem.*, **136**, 229 (1940).

LETTERS TO THE EDITORS

THE ISOLATION OF ETIOCHOLANOL-3(α)-DIONE-11,17 FROM HUMAN URINE

Sirs:

We have recently ascertained the structure of a steroid isolated from human urine which has hitherto not been described from that source. The compound has proved to be etiocholanol-3(α)-dione-11,17. It was obtained from the urine of normal men and women in amounts corresponding to an excretion of 1 to 2 mg. per 24 hours. It was likewise isolated from the urine of patients with the following disorders: cancer of the breast, cancer of the prostate, lymphatic leucemia, adrenogenital syndrome, and Cushing's syndrome. In view of the fact that this compound possesses an oxygen substituent in the 11 position which is characteristic of many of the adrenal cortical steroids, it can in all probability be considered a normal metabolic product of one of more of the adrenal cortical hormones.

The compound was isolated from the neutral ketonic fraction of hydrolyzed urine by chromatographic separation. It was eluted from alumina with ether containing 0.5 per cent methanol or from magnesium silicate with ether-benzene (1:3) mixtures. The compound melted at 188–189°; $[\alpha]_D^{27} = +95.8° \pm 3°$ (0.34 per cent in ethanol); $C_{19}H_{28}O_3$, calculated, C 74.97, H 9.27; found, C 75.00, 75.28, H 9.54, 9.29. It formed a monoacetate melting at 163–164°; $[\alpha]_D^{13} = +145.4° \pm 2°$ (0.563 per cent in ethanol); $C_{21}H_{30}O_4$, calculated, C 72.81, H 8.72; found, C 72.65, H 8.71. The monoacetate was recovered unchanged after treatment with CrO_3 in acetic acid at room temperature. Oxidation of the unesterified compound under the same conditions yielded etiocholantrione-3,11,17, m.p. 132–133°; $[\alpha]_D^{13} = +148.5° \pm 1°$ (1.018 per cent in ethanol); $C_{19}H_{26}O_3$, calculated, C 75.46, H 8.67; found, C 75.38, H 8.74.

The etiocholanol-3(α)-dione-11,17 and the corresponding monoacetate were identified by direct comparison of the melting point of mixtures and of the infra-red spectra with the compounds prepared by Dr. L. H. Sarett by partial synthesis from desoxycholic acid.[1] They were in all respects

[1] Sarett, L. H., *J. Biol. Chem.*, **162**, 619 (1946).

identical. We wish to thank Dr. Sarett for samples generously made available to us.

Memorial Hospital
  Sloan-Kettering Institute for Cancer Research
  New York

SEYMOUR LIEBERMAN
KONRAD DOBRINER

THE ENZYMATIC FORMATION AND THE ACCUMULATION OF LARGE AMOUNTS OF A METAPHOSPHATE IN BAKERS' YEAST UNDER CERTAIN CONDITIONS*

Sirs:

Jenner and Brachet reported[1] that the affinity of yeast cells for basic dyes is strongly enhanced when yeast is incubated in phosphate and glucose-containing media following several passages through nutrient solutions devoid of phosphate. This enhancement is accompanied by a corresponding increase of the amount of the acid-insoluble, non-lipide P fraction. The authors suggested the hypothesis that this behavior of the yeast cells was caused by the enzymatic synthesis of ribonucleic acid.

Formation of Acid-Insoluble Non-Lipide P Compound in Bakers' Yeast

100 gm. of Fleischmann's yeast were suspended in 5 liters of nutrient solution and aerated at room temperature. Solution A, 0.5 per cent $(NH_4)_2SO_4$ + 0.07 per cent KCl + 0.035 per cent $MgSO_4$ + 0.05 N succinate buffer (pH 5.2) + 1 per cent glucose. Solution B, Solution A (without succinate) + 0.1 N sodium phosphate mixture (pH 5.2).

Preliminary incubation	Final incubation	Acid-insoluble non-lipide P in 100 gm. moist yeast	
		Before final incubation	After final incubation
		mg.	*mg.*
12 hrs. in Solution A	2 hrs. in Solution B	160	1250
1 hr. " " "	2 " " " "	153	600
None	2 " " " "	145	315

Since we were interested in the metabolism of nucleic acids, we investigated the behavior of the acid-insoluble P fraction of the yeast under conditions similar to those of the Belgian authors. We observed likewise a striking increase of the amount of this fraction. Some typical results are recorded in the accompanying table.

The phosphate compound can be extracted from the "P-enriched" yeast by cold water after a preliminary treatment of the yeast with cold alcohol and ether. It is completely precipitated from the aqueous extract by the addition of barium acetate at pH 4. The dried reprecipitated Ba salt contains 18.7 per cent P and 41.7 per cent Ba (P:Ba = 2:1). It is free of nitrogen, ribose, and inorganic phosphate. On heating it in N HCl at 100°

[1] Jeener, R., and Brachet, J., *Enzymologia*, 11, 222 (1944).

775

during 7 minutes, 85 to 90 per cent of its P is converted to orthophosphoric acid. The free phosphorous compound coagulates a solution of egg white.

According to these observations the P compound is a metaphosphate.[2]

Since metaphosphate present in cells appears in the nucleic acid fractions obtained according to the method of Schmidt and Thannhauser,[3] the determinations of nucleic acid P should be supplemented by ribose or purine determinations whenever the possible presence of metaphosphate is indicated by the detection of easily hydrolyzable P in the nucleic acid fraction. We found by the application of those criteria that no appreciable amounts of metaphosphate are present in animal tissues under ordinary conditions.

Boston Dispensary   GERHARD SCHMIDT
  Tufts College Medical School   LISELOTTE HECHT
  Boston   S. J. THANNHAUSER

---

[2] After the completion of this report, a paper on the same subject by Wiame came to our attention (Wiame, J. M., *Bull. Soc. chim. biol.*, **28**, 552 (1946)). Wiame concludes from his observations, which agree essentially with our chemical findings, that a metaphosphate is formed and accumulated in yeast. Mann (Mann, T., *Biochem. J.*, **38**, 345 (1944)) demonstrated the presence of metaphosphate in *Aspergillus niger*.

[3] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).

BACTERIAL SYNTHESIS OF AN AMYLOPECTIN-LIKE POLYSACCHARIDE FROM SUCROSE*

Sirs:

We have found that sucrose can be converted to a glycogen- or amylopectin-like polysaccharide by certain bacteria of the *Neisseria* genus. Dextrans and levans have long been known to be formed directly from sucrose, but polysaccharides of the starch or glycogen class are generally believed to require glucose-1-phosphate as substrate.

The present polysaccharide, obtainable in amounts as large as 3 to 5 gm. per liter from cultures grown in 5 per cent sucrose broth, is soluble in cold water but not in 50 per cent alcohol. Its solutions are highly opalescent and stain purple-red with iodine and both these properties are rapidly lost upon treatment with saliva. A representative specimen had $[\alpha]_D^{20} = +185°$ ($c = 0.2$ in 0.5 N NaOH) and less than 0.1 per cent of the reducing power of glucose. Its rate of hydrolysis in 1.0 N HCl at 100° was the same as that of samples of glycogen and amylopectin and was 4 to 5 times that of *Leuconostoc* dextran; the acid hydrolysate, corrected for the entry of water, had $[\alpha]_D^{20} = +52.5°$ and 94.4 per cent reducing sugars as glucose. Hydrolysis was obtained also with α- and with β-amylase to the extent respectively of 0.82 and 0.43 mg. of reducing sugar (expressed in terms of maltose) per 1.0 mg. of polysaccharide. Furthermore, phosphorolysis occurred when the polysaccharide was incubated with potato phosphorylase and inorganic phosphate.

The polysaccharide is derived from sucrose: when washed bacterial cells obtained from glucose broth cultures were incubated with 0.05 M solutions of various sugars in maleate buffer, pH 6.4, abundant amounts of the polysaccharide were formed in the test mixture with sucrose but none at all in the test mixtures with glucose, glucose and fructose, maltose, lactose, trehalose, α-methylglucoside, melibiose, raffinose, or melezitose.

When glucose-1-phosphate was used as substrate, traces of iodine-coloring polysaccharide were formed. However, this slight capacity, observed in test mixtures containing low concentrations (<0.0003 M) of inorganic phosphate, was entirely suppressed in mixtures in which the ratio of the molecular concentration of inorganic phosphate to that of the substrate was increased to 6.0 or above. In contrast, the capacity of the bacteria to form abundant polysaccharide from sucrose was not inhibited at all by the greatly increased concentration of inorganic phosphate. Hence, the synthesis of amylopectin-like polysaccharide when sucrose is the substrate

apparently can be accomplished without the participation of glucose-1-phosphate as an intermediate substance.

The synthesis of amylopectin-like material from sucrose by the present bacteria appears remarkably like the synthesis of dextran from sucrose by *Leuconostoc* enzymes,[1, 2] which likewise does not require glucose-1-phosphate as an intermediate substance.[2]

The equation

$$nC_{12}H_{22}O_{11} \longrightarrow (C_6H_{10}O_5)_n + nC_6H_{12}O_6$$

Sucrose   polyglucoside   fructose
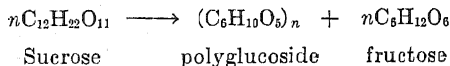
would seem to express both the synthesis of dextran and of the present polysaccharide. Although the latter synthesis has not yet been observed independently of the bacterial cells, we would suggest that the name *amylosucrase* be given to the enzyme system in *Neisseria* sp. responsible for the conversion of sucrose to the glycogen- or amylopectin-like polysaccharide in order to distinguish it from the phosphorylase plus "cross-linking" enzyme systems from animal tissues[3] or from potatoes[4] which convert glucose-1-phosphate to glycogen or amylopectin.

Department of Bacteriology and Immunology   EDWARD J. HEHRE
   Cornell University Medical College   DORIS M. HAMILTON
   New York City

[1] Hehre, E. J., *Science*, **93**, 237 (1941); *J. Biol. Chem.*, **163**, 221 (1946).

[2] Hehre, E. J., *Proc. Soc. Exp. Biol. and Med.*, **54**, 240 (1943).

[3] Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **151**, 57 (1943).

[4] Haworth, W. M., Peat, S., and Bourne, E. J., *Nature*, **154**, 236 (1944).

MODIFICATIONS IN THE LACTOBACILLUS FERMENTI 36 ASSAY FOR THIAMINE*

Sirs:

A previous publication[1] has outlined the use of *L. fermenti* 36 for the assay of thiamine. Subsequent experience in this laboratory with several hundred assays has revealed certain difficulties in using the method, which are in the main associated with the growth of inoculum cultures and the development of standard curves with desirable low blank values.

Growth of inocula in the prescribed medium,[1] which includes 0.1 γ of thiamine per 10 ml., and frequent transfer of the organism in stab cultures which contain no additional thiamine result in the development of standard curves with excessively high blank values. Apparently the organism develops the ability to synthesize thiamine. This is illustrated in Table I

TABLE I

Thiamine per 10 ml.	Optical density	
	1st transfer	30th transfer
γ		
0	0.040	0.375
0.01	0.290	0.550
0.02	0.440	0.660
0.03	0.550	0.700
0.05	0.640	0.730

where the response of *L. fermenti* to thiamine is observed after 30 daily transfers on stock agar.

It was felt that the addition of extra thiamine to the agar tubes and inoculum cultures might repress the synthesis of the vitamin by the organism and maintain it in its dependent state. The results in Table II indicate that this is the case. Thiamine is present at a level of 10 γ per tube, which is considered a 100-fold excess over the organism's optimum requirements. After thirty-one transfers the culture grown in extra thiamine is still useful for assay, whereas the one transfered on stock agar is less desirable.

Low blank values are also favored by centrifuging the inoculum, resuspending in 10 ml. of sterile saline, and finally, diluting 1 drop of this suspension in 25 ml. of saline rather than in 10 ml. as previously described.

[1] Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **155**, 153 (1944).

TABLE II

Thiamine per 10 ml.	Optical density			
	1st transfer		31st transfer	
	I	II	I	II
γ				
0	0.140	0.023	0.185	0.100
0.01	0.430	0.410	0.345	0.470
0.02	0.540	0.550	0.420	0.590
0.03	0.630	0.600	0.470	0.650
0.05	0.720	0.720	0.520	0.690

I, inoculum culture grown in basal medium[1] + 0.1 γ of thiamine per tube; stab culture grown on stock agar. II, inoculum culture grown in basal medium + 10 γ of thiamine per tube; stab culture grown on agar + 10 γ of thiamine per tube.

Routine assays during the past 6 months with the modified procedure have given consistent satisfactory performance.

Department of Chemistry   VERNON H. CHELDELIN
  Oregon State College   MARGARET JEAN BENNETT
  Corvallis   HARRY A. KORNBERG

THE UTILIZATION OF FORMATE IN URIC ACID SYNTHESIS

Sirs:

In a continuation of studies on the biological precursors of uric acid carbon, sodium formate containing 3.34 atoms per cent excess C^{13} has been administered to pigeons at the rate of 0.75 mM per hour for 12 hours. Uric acid excreted during this time was extracted and purified. Methods of degradation of uric acid by alkaline MnO_2 have been outlined briefly in the previous publication.[1] CO_2 formed by this degradation is derived from carbon 6, urea from carbons 2 and 8, and glyoxylic acid from carbons 4 and 5. In this experiment glyoxylic acid was not further degraded to separate carbons 4 and 5. The atoms per cent excess C^{13} present in these fractions of uric acid degradation were as follows: carbon 6, 0.01; carbons 2 and 8, 2.41; carbons 4 and 5, 0.10. A sample of respiratory CO_2 taken at the mid-point of the experiment contained 0.02 atom per cent excess C^{13}. Although administered formic acid carbon did not appear in the expired CO_2 to a significant extent, formic acid is a precursor of the ureide carbons of uric acid and to a lesser degree of the 2-carbon fraction containing carbons 4 and 5. From the above data it may be calculated that 72 per cent of the ureide carbons of uric acid excreted was derived from formic acid administered. It is thus seen that formate may play an important rôle in avian metabolism. Previous studies[1] have demonstrated that the carboxyl carbon of acetate is likewise an important precursor of the ureide carbons but that CO_2 and the carboxyl carbon of glycine are not. These facts would suggest that CO_2 is not reduced to formate in the bird but that formate may be derived from the carboxyl carbon of acetate. This experiment also demonstrates that formate is not a precursor of carbon 6 of uric acid. It is believed that this carbon atom is derived solely from CO_2.

Department of Physiological Chemistry
  School of Medicine
  University of Pennsylvania
  Philadelphia

JOHN M. BUCHANAN
JOHN C. SONNE

[1] Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **166**, 395 (1946).

STREPOGENIN ACTIVITY OF SERYL GLYCYL GLUTAMIC ACID

Sirs:

Since Plattner and Clauson-Kaas[1] have isolated the tomato-wilting agent elaborated by the pathogenic fungus *Fusarium lycopersici*, and have shown it to be a peptide containing aspartic acid, the idea occurred to the author that this toxic substance might be an inhibitory structural analogue of strepogenin.[2, 3] This working hypothesis arose because considerable evidence had been found in this laboratory to show that strepogenin is a peptide of glutamic acid. The arguments for this cannot be expanded at this time, except to say that strepogenin concentrates are rich in glutamic acid, and that, for *Lactobacillus casei*, derivatives of glutamic acid such as glutamine and glutathione have activity. However, these γ-amides of glutamic acid are destroyed by heat, whereas strepogenin is not.

With either crude or pure preparations of the tomato-wilting *Fusarium* substance (lycomarasmin),[4] it has been possible to show that the biological effects of the toxin are negated by concentrates of strepogenin. Thus the wilting action on tomato leaves and also the inhibition of growth of *L. casei* caused by lycomarasmin were reversed by strepogenin preparations. The antagonism was competitive, at least over a limited range of concentrations.

The elucidation of the structure of lycomarasmin therefore becomes of prime importance, because if this were known, the constitution of strepogenin might be deduced from it. Plattner and Clauson-Kaas[4] found that hydrolysis of lycomarasmin, $C_9H_{15}O_7N_3$, yielded aspartic acid, glycine, and pyruvic acid, and proposed a structure for it.[4] Because certain serine peptides are known to yield pyruvic acid on acid hydrolysis, it seemed possible that lycomarasmin might really contain serine. Accordingly, seryl glycyl aspartic acid and glycyl seryl aspartic acid were synthesized and found to possess tomato leaf-wilting activity. The first was about one-sixth, and the second was one-half to one-fourth as active as lycomarasmin. Despite this activity it is uncertain whether either of these peptides is identical with lycomarasmin because they were less potent and more water-soluble. These differences, however, may have resided in the fact that the serine was *dl*.

Nevertheless, seryl glycyl glutamic acid was synthesized and tested for strepogenin activity. The synthesis was accomplished by the following

[1] Plattner, P. A., and Clauson-Kaas, N., *Helv. chim. acta*, **23**, 188 (1944).

[2] Woolley, D. W., *J. Exp. Med.*, **73**, 487 (1941). Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, **67**, 1734 (1945).

[3] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).

[4] Plattner, P. A., and Clauson-Kaas, N., *Experientia*, **1**, 1 (1945).

stages: tosyl serine ethyl ester → hydrazide → azide → tosyl seryl glycine ethyl ester → hydrazide → azide → tosyl seryl glycyl diethyl glutamate → free acid → seryl glycyl glutamic acid. This tripeptide, in which serine was *dl*, possessed relative strepogenin activity of 1 for *L. casei*.[3] One would expect the optically active form to be twice as potent. Furthermore, like natural strepogenin, and in contrast to glutamine or glutathione, the activity was not affected by heating. Finally, this peptide antagonized the tomato-wilting action of seryl glycyl aspartic acid.

The strepogenin activity of seryl glycyl glutamic acid was not great enough to justify a conclusion that it was identical with the naturally occurring substance. Concentrates of the latter with relative potency of 40 have been made. By determination of diffusion rate the molecular weight of natural strepogenin was found to lie in the neighborhood of 300 to 500. It is possible that seryl glycyl glutamic acid may be a fragment or relative of strepogenin, and as such has partial activity.

Aside from any hypothesis the following facts stand out: (1) seryl glycyl aspartic acid and glycyl seryl aspartic acid have lycomarasmin activity, and (2) seryl glycyl glutamic acid has strepogenin activity for *L. casei*. The former and the latter peptides are antagonistic to each other in both types of test.

Laboratories of The Rockefeller Institute for Medical Research   D. W. WOOLLEY
New York

INDEX TO AUTHORS

INDEX TO SUBJECTS

793